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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/12, C07K 14/705, C12N 5/10, C12Q 1/68, C07K 16/18, G01N 33/68, A61K 38/17

A1

(11) International Publication Number: WO 98/33905

(43) International Publication Date: 6 August 1998 (06.08.98)

(21) International Application Number: PCT/GB98/00285

(22) International Filing Date: 30 January 1998 (30.01.98)

(30) Priority Data:
9701867.5 30 January 1997 (30.01.97) GB

(71) Applicant *(for all designated States except US)*: ISIS INNOVATION LIMITED [GB/GB]; 2 South Parks Road, Oxford OX1 3UB (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: ASHCROFT, Frances, M. [GB/GB]; Stonecroft, Brill Road, Horton–cum–Studley, Oxford OX33 1BX (GB).

(74) Agents: O'BRIEN, Caroline, J. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).

(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: K–ATP CHANNELS, MATERIALS AND METHODS RELATING TO CELL MEMBRANE PROTEINS

(57) Abstract

This invention concerns materials and methods relating to an ATP–sensitive potassium ion channel (a K–ATP channel) molecule which is implicated in a number of disease states associated with abnormal coupling of cellular metabolism to K^+ fluxes and/or electrical activity. In particular the invention provides variant forms of the Kir6.2 protein subunit of a K–ATP channel. These variant forms function as a K–ATP channel in the absence of association with a sulphonylurea receptor (SUR) protein and have great value in the screening for drugs which interact with a K–ATP channel and the identification of the K–ATP channel sites at which such drugs interact.

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K-ATP CHANNELS, MATERIALS AND METHODS RELATING TO CELL MEMBRANE PROTEINS

The present invention concerns materials and methods relating to cell membrane proteins. The cell membrane proteins of particular interest relate to ATP-sensitive potassium ion channel (a K-ATP channel) molecules, subunits and variants thereof.

K-ATP channels are found in many tissues where they play important roles in both physiological and pathophysiological conditions. Basically they couple cell metabolism to electrical activity and play important roles in the physiology and pathophysiology of many tissues. Examples of physiological conditions include insulin secretion, whereas pathophysiological conditions include cardiac and cerebral ischaemia. These tissues include: pancreatic β-cells; smooth, skeletal and cardiac muscle; neurones, including those of the cortex, hippocampus, neocortex, olfactory bulb, cerebellum, substantia nigra (pars reticulata and pars compacta) and respiratory neurones; axons; epithelial cells including kidney tubules. The functional role of the K-ATP channel has been reviewed previously (Ashcroft F.M. (1988) Ann Rev. Neurosci 11, 97-118, Ashcroft F.M. et al., (1990) Cell Sig. 2, 197-214). Its particular role in the pancreatic β-cell has been described (Ashcroft F.M. and Rorsman, P., Prog. Biophys. Mol. Biol. (1990) 54, 87-143) and also it has been found to be effective in substantia nigra neurones, cardiac muscle and smooth muscle (Quayle et al., Physiol. Rev (1997) 77, 1165-1232; Nichols & Lederer Am. J. Physiol. (1991) 261, H1675-H1686).

In pancreatic β-cells, K-ATP channels mediate insulin secretion both in response to glucose (the primary physiological stimulus) and to clinically important drugs. The effects of glucose are mediated indirectly, as a consequence of its metabolism, whereas drugs interact directly with the channel. Metabolic regulation of β-cell K-ATP channels is thought to be mediated by changes in intracellular ATP and MgADP levels which inhibit and activate the channel, respectively. Glucose metabolism elevates intracellular ATP and concomitantly lowers intracellular ADP. This closes K-ATP channels in the β-cell plasma membrane, producing a membrane depolarisation which activates voltage-dependent Ca^{2+} channels, enhances Ca^{2+} influx and triggers insulin release.

The most characteristic property of K-ATP channels is that they are inhibited by an increase in the intracellular ATP concentration. They may also be regulated by hormones and transmitters (as in smooth muscle) Quayle et al., 1997 supra. K-ATP channels are inhibited by sulphonylurea drugs (such as glibenclamide and tolbutamide) which are used in the treatment of non-insulin-dependent diabetes mellitus (Ashcroft F.M. and Ashcroft S.J.H., Biochem Biophys Acta 1175, 45-49 (1992))

to stimulate insulin release. These drugs bind to the sulphonylurea receptor subunit of the K-ATP channel (SUR eg SUR1 or SUR2) which interacts with the pore subunit (eg Kir6.2) of the K-ATP channel to bring about its closure, and thereby stimulate insulin secretion. In contrast, other drugs such as diazoxide which also interact with SUR act as K-ATP channel openers and therefore inhibit insulin release. Diazoxide has been used to treat persistent hyperinsulinaemic hypoglycaemia of infancy, a disease associated with unregulated insulin secretion.

Since the β-cell K-ATP channel plays a central role in glucose-stimulated insulin secretion, it is likely that mutations in this channel might be associated with diabetes mellitus. K-ATP channels also play important roles in the response to cardiac and cerebral ischaemia and may be implicated in a number of disease states detailed more fully below where there is an abnormal coupling of cellular metabolism and electro-physiological activity.

The regulation of the β-cell K-ATP channel by adenine nucleotides is extremely complex and probably involves several different sites of action. In addition to its well-known inhibitory effect, MgATP enhances K-ATP channel activity as evidenced by the fact that when MgATP is removed, channel activity is greater than that recorded in the control solution prior to application of the nucleotide. This 'refreshment' of channel activity is not observed in the absence of Mg^{2+}, nor is it supported by non-hydrolysable ATP analogues, indicating that MgATP hydrolysis is required. ADP also has both stimulatory and inhibitory actions. In the absence of Mg^{2+}, ADP blocks channel activity. When Mg^{2+} is present, however, high concentrations of ADP are inhibitory whereas low concentrations potentiate channel activity. This suggests that MgADP both activates and inhibits channel activity and that the inhibitory effect dominates in Mg-free solutions or at high MgADP concentrations. There is evidence that nucleotides also modulate the response of the K-ATP channel to drugs. The K-channel opener diazoxide, for example, antagonises the inhibitory effects of MgATP on the β-cell K-ATP channel. In β-cells, the drug has no effect, or is even inhibitory, in the absence of internal Mg^{2+} or when ATP is replaced by non-hydrolysable ATP analogues. This result has been used to support the idea that the action of diazoxide requires protein phosphorylation. However, diazoxide is also effective in the presence of hydrolysable ADP, suggesting that it is more likely that the effect of the drug requires nucleotide hydrolysis rather than phosphorylation. Trapp, S. et al., (1997) Proc. Natl. Acad-Sci., USA Vol 94 pp8872-8877 reports on activation and inhibition of K-ATP currents by guanine nucleotides. They used deletion mutant forms of Kir6.2 as provided

herein and which express independently of SUR1 to show that GTP blocks K-ATP currents by interaction with Kir6.2 and that the potentiatory effects of GTP are endowed by SUR1.

Cloning of a 140kD high-affinity sulphonylurea receptor (SUR1) from insulinoma cells revealed it to be a member of the ATP-binding cassette (ABC) transporter family, with 2 nucleotide binding folds (Aguilar-Bryan L. et al., 1995 Science, 268, 423-425).

As a result of electrophysiological studies (Ämmälä,C. et al., 1996 J. Physiol. 494,3, 709-714), it has been shown that SUR1 does not possess intrinsic channel activity. Instead, it endows sulphonylurea sensitivity on several types of inwardly-rectifying K-channels.

The properties of the K-ATP channel indicate that it belongs to the superfamily of inwardly-rectifying potassium channels or Kir channels (Doupnik C.A. et al., Curr. Op. Cell Biol. (1995) 5, 268-278). At least six distinct subfamilies of Kir channels have been cloned. Like these cloned channels, the native K-ATP channel shows inward rectification, little time- or voltage dependence, and is strongly K-selective (Ashcroft and Ashcroft et al., supra). It has been suggested previously that a number of Kir channels that have been cloned constitute the K-ATP channel including Kir1.1a (Ho K. et al., Nature, 362, 31-39 (1993) and Kir3.4 (Ashford M.L.J. et al., Nature, 370, 456-459 (1994)). A further paper reporting the cloning of a K-ATP channel (Kir6.1) is Inagaki et al., J Biol. Chem. 270, 5691-5694 (1995). However none of these Kir channels have a tissue distribution similar to that of the native K-ATP channel.

The present inventors found that both Kir6.1 and Kir1.1a can couple with SUR1. They used Kir6.1 and Kir1.1a clones as probes to screen a murine β-cell cDNA library. The approach resulted in the cloning of a mouse gene for an inwardly-rectifying K-channel now designated as Kir6.2. In WO97/18308 the present inventors provide for both murine and human Kir6.2 nucleotide sequences and amino acid sequences ascribed thereto. The sequence information is provided herein as figures 1 and 2. Reference may be made to WO97/18308 for further details about the properties of Kir6.2.

In the text reference is made to the drawings listed below. A general note of explanation may be helpful here. In order to study K-ATP currents, patch-clamp recordings from isolated inside-out membrane patches are performed. These are produced by bringing a patch-recording electrode up to the surface of the cell and forming a high- resistance seal between the glass wall of the electrode and the cell membrane. This isolates a small patch of membrane under the pipette tip: the

currents flowing through ion channels in this membrane patch can then be recorded. If the patch is moved away from the cell, the membrane is then excised (and remains spanning the tip of the patch electrode). This is known as an inside-out patch because it has its intracellular membrane surface exposed to the bath solution. The effects of agents (drugs, cytosolic constituents etc) which act from the inside of the membrane can then be tested simply by applying them to the bath solution (the patch electrode is fabricated from a hollow glass capillary and is filled with a highly conductive salt solution).

Figure 1. Is the nucleotide sequence of the murine Kir6.2 gene and the amino acid sequence ascribed to it.

Figure 2. Is the nucleotide sequence of the human Kir6.2 gene and the amino acid sequence ascribed to it. The boxed amino acids are those which are different to the amino acids at equivalent residues in the murine sequence shown in Figure 1.

Figure 3. Is a representation of a K-ATP channel showing the octameric complex of Kir and SUR subunits (eg Kir6.2 and SUR1).

Figure 4. Shows the nucleotide and amino acid sequence for (a) Kir6.2ΔC26 or (b) Kir6.2ΔC36. The boxed amino acids are those which are different to the amino acids at equivalent residues in the murine sequence shown in Figure 1.

Figure 5. Shows effects of deleting residues in the C-terminus of Kir6.2 on K-ATP channel activity.

Figure 6. Shows the effects of deleting residues in the C-terminus of Kir6.2 on the sensitivity of K-ATP channel activity to ATP.

Figure 7. Shows the putative topology of Kir6.2 showing the location of the mutations and polymorphisms in human Kir6.2.

Figure 8. Shows the predicted amino acid sequence of Kir6.2 and comparison with other members of the inward rectifier family. Residues shared with at least Kir6.1 are boxed; putative transmembrane (TM) and pore (H5) domains are overlined. Dashes represent minimal gaps introduced to maximise the comparison.

Figure 9 A. Schematic drawing of Kir6.2 with the location of the transmembrane domains (M1,M2) and the C-terminal deletions marked Kir6.2ΔC26 = ΔC26 and Kir6.2ΔC36 = ΔC36. B. Whole cell currents recorded from an oocyte injected with mRNA encoding wtKir6.2 or Kir6.2ΔC26 in response to a series of voltage steps from -120mV to +20mV before and

15 min after exposure to 3mM azide.
C. Whole-cell current-voltage relations recorded from an oocyte injected with mRNA encoding wtKir6.2 (open symbols) or Kir6.2ΔC26 (filled symbols), before (□,■) and after (o,•) exposure to 3mM azide. Same oocytes as in B.
D. Mean whole-cell currents recorded at -100mV before (filled bars) and 10 min after (hatched bars) exposure to 3mM azide from oocytes injected with mRNA encoding wtKir6.2, Kir6.2ΔC26, Kir6.2ΔC36, Kir6.2ΔC26 + SUR1. The number of oocytes is given above the bars.

Figure 10 A. Single-channel currents recorded at -60mV from an inside-out patch excised from an oocyte injected with mRNA encoding Kir6.2ΔC26, or Kir6.2ΔC26 + SUR1.
B. Mean single-channel current-voltage relations recorded for Kir6.2ΔC26 (o) or Kir6.6ΔC26 + SUR1 (•).

Figure 11 A. Macroscopic currents recorded from 2 different inside-out patches in response to a series of voltage ramps from -110mV to +100mV. Oocytes were injected with mRNAs encoding Kir6.2ΔC26 or Kir6.2ΔC26 + SUR1. 1mM ATP was added to the internal solution as indicated by the bar.
B. Mean macroscopic current amplitudes at -100mV before (filled bars) and after (hatched bars) patch excision from oocytes injected with the mRNAs indicated. The number of oocytes is given above the bars.
C. Mean macroscopic slope conductance recorded with the intracellular additions indicated, expressed as a percentage of the slope conductance in control solution (no additions) for MgADP and tolbutamide (Tb) data. Diazoxide (Dz) was added in the presence of 100μM MgATP and is expressed as a percentage of the current amplitude in 100μM MgATP solution. The dashed line indicates the current level in the absence of the test compound. The number of oocytes is given above the bars.
D. Mean ATP dose-response relationships for Kir6.2ΔC26 currents (o, n=7) and Kir6.2ΔC26/SUR1 currents (•, n=6). Test solutions were alternated with control solutions and the slope conductance (G) is expressed as a percentage of the mean (G_c) of that obtained in control solution before and after exposure to ATP. Conductance was measured between -20 and -100mV and is the mean of 5 voltage ramps. The lines are the best fit of the data to the Hill equation using the mean values for K_i (106μM) and for the Hill coefficient (1.2).

Figure 12 A. Whole-cell currents recorded from an HEK293 cell transfected with cDNA encoding Kir6.2ΔC36 in response to a series of voltage steps from -110mV to +30mV, immediately after forming the whole-cell configuration and ~10 min later. The pipette contained 0.3mM ATP.
B. Mean whole-cell current amplitudes recorded from HEK293 cells transfected with cDNA encoding Kir6.2Δ36 immediately after forming the whole-cell configuration

(filled bars) and ~10 min later (hatched bars). Cells were dialysed with the ATP concentrations indicated.

Figure 13. Macroscopic currents recorded from 2 different inside-out patches in response to a series of voltage ramps from -110mV to +100mV. Oocytes were injected with mRNAs encoding Kir6.2ΔC26 (above) or Kir6.2ΔC26 + SUR1 (below). 100μm ADP, 340μM diazoxide or 100μM tolbutamide were added to the internal solution as indicated by the bars.

Figure 14. Mean macroscopic conductance recorded with the intracellular additions indicated, expressed as a percentage of the conductance in control solution (no additions) for MgADP and tolbutamide data. Diazoxide was added in the presence of 100μM MgATP and is expressed as a percentage of the current amplitude in 100μM MgATP solution. The dashed line indicates the current level in the absence of the test compound. Oocytes were injected with mRNAs encoding Kir6.2ΔC26, Kir6.2ΔC26 + SUR1, Kir6.2ΔC36 or Kir6.2ΔC36 + SUR1 as indicated.

Figure 15. Effects of the imidazolines phentolamine and efaroxan on wt Kir6.2+SUR1 currents when applied to the outer membrane surface.
A. Whole-cell currents recorded from an oocyte coinjected with mRNA encoding Kir6.2 and SUR1 in the presence of 3mM azide. Efaroxan (30μM, closed circle and 100μM, closed square) was added and removed from the extracellular solution as indicated. Control is shown as open circle.
B. Mean steady-state whole-cell currents recorded at 100mV in response to the drugs indicated, expressed as their value in control solution prior to the addition of the drug.

Figure 16. Effects of the imidazolines phentolamine and efaroxan on wt Kir6.2+SUR1 currents when applied to the inner membrane surface.
Macroscopic currents recorded an inside-out patch excised from an oocyte expressing wt Kir6.2+SUR1. Currents were recorded in response to a series of voltage ramps from -110mV to +100mV (holding potential, 0mV). Phentolamine (30μM, A) or efaroxan (30μM, B) were added to the internal solution as indicated by the bar.

Figure 17. Effects of the imidazoline phentolamine on Kir6.2ΔC36 currents.
Macroscopic current recorded an inside-out patch excised from an oocyte expressing Kir6.2ΔC36. Currents were recorded in response to a voltage ramp from -110mV to +100mV (holding potential, 0mV) in the absence (control) or presence of phentolamine (10μM phentolamine) as indicated.

Figure 18. Effects of the imidazoline efaroxan on

Kir1.1a currents.
Macroscopic current recorded an inside-out patch excised from an oocyte expressing Kir1.1a. Currents were recorded in response to a voltage ramp from -100mV to +100mV (holding potential, 0mV) in the absence (control) or presence of efaroxan (30μM to 1mM) as indicated.

Figure 19. Single channel currents recorded from an inside-out patch excised from a Xenopus oocyte injected with the mRNA encoding Kir6.2 and SUR1 (top 2 traces) or with Kir6.2ΔC26 (lower 2 traces). Currents were recorded first in control solution and then after the addition of 1μM oleoyl CoA. The pipette solution contained (mM): 140 Kcl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 10 HEPES (pH 7.4 with KOH) and the internal (bath) solution contained (mM): 110KCL, 1.44 $MgCl_2$, 30 KOH, 10 EGTA, 10 HEPES (pH 7.2 with KOH). Holding potential -60mV.

Figure 20. Macroscopic currents recorded from a giant patch on an oocyte injected with Kir6.2ΔC36. The holding potential was 0 mV and the voltage was successively ramped from -110 mV to +100 mV over a 3 s period. The patch was excised into ATP-free solution at the arrow. 1 mM ATP was subsequently added to the intracellular solution as indicated.

Figure 21.
A. Macroscopic currents recorded from 3 different inside-out patches in response to a series of voltage ramps from -110 mV to +100 mV. Oocytes were injected with mRNAs encoding Kir6.2ΔC36, Kir6.2ΔC26-E179Q, Kir6.2ΔC26-I167M or Kir6.2ΔC26-R50G. 1 mM ATP was added to the internal solution as indicated.
B. Mean ATP dose-response relationships for Kir6.2ΔC36 currents (•,n=11), Kir6.2ΔC26-E179Q currents (O, n=6), Kir6.2ΔC26-I167M currents (■, n=8) and Kir6.2ΔC26-R50G currents (□, n=8). Test solutions were alternated with control solutions and the slope conductance (G) is expressed as a percentage of the mean (G_c) of that obtained in control solution before and after exposure to ATP. Conductance was measured between -20 and -100 mV and is the mean of 5 voltage ramps. The lines are the best fit of the data to the Hill equation using the mean values for K_i and h given in the text.

Figure 22.
A. Macroscopic currents recorded from 4 different patches in response to a series of voltage ramps from -110 mV to +100 mV. Oocytes were injected with mRNAs encoding Kir6.2ΔC36, Kir6.2ΔC26-E179Q, Kir6.2ΔC26-E167M or Kir6.2ΔC26-R50G. The patch was excised into ATP-free solution at the arrow.
B. Relationship between the increase in conductance on patch excision and 1 / K_i for channel inhibition by ATP, for the channels indicated. The conductance following excision is expressed as a fraction of that in the cell-

attached configuration. The line is drawn to the equation $G/G_c = 1 / (1+([ATP]/K_i))$.

Figure 23.
A. Macroscopic currents recorded from 3 different inside-out patches in response to a series of voltage ramps from -110 mV to +100 mV. Oocytes were injected with mRNA encoding Kir6.2ΔC36. 1 mM ATP, 1 mM ADP or 1 mM AMP were added to the internal solution as indicated.
B. Mean dose-response relationships for Kir6.2ΔC36 currents exposed to ATP (O, n=11), ADP (■, n=5) or AMP (□, n=6), as indicated. Test solutions were alternated with control solutions and the slope conductance (G) is expressed as a percentage of the mean (Gc) of that obtained in control solution before and after exposure to ATP. The lines are the best fit of the data to the Hill equation using the mean values for K_i and h given in the text.

The present application is concerned with materials and methods relating to the inventors' new and surprising discoveries about K-ATP channels, particularly the Kir6.2 subunit thereof summarised below.

1. Modified forms of Kir6.2 which lack a number of C-terminal amino acid residues are able to express functional currents in the absence of SUR1.

2. Kir6.2 has as imidazoline binding site at a polypeptide location which in the membrane lies intracellularly. Further that the binding site must include residues which are different in Kir6.2 and Kir1.1a.

These disclosures are discussed more fully below.

ATP-sensitive potassium channels (K-ATP channels) couple cell metabolism to electrical activity and play important roles in the physiology and pathophysiology of many tissues. For example, in pancreatic β-cells, K-ATP channels link changes in blood glucose concentration to insulin secretion. They are also the target for clinically important drugs such as sulphonylureas, which stimulate secretion, and the K-channel opener diazoxide, which inhibits insulin release.

The K-ATP channel is an octameric complex of two proteins: an inward rectifier K-channel subunit, Kir6.2 and the sulphonylurea receptor SUR which assemble in a 4:4 stoichiometry to form an octameric channel. The structure is shown in Figure 3. Both Kir6.2 and SUR are required to form a functional K-ATP channel and a single unattached Kir6.2 subunit within the whole complex is sufficient to inhibit its activity. Unlike most other Kir channels, Kir6.2 does not yield functional currents alone, but only when coexpressed with SUR1. Therefore one

has not been able to ascertain which properties of the K-ATP channels are intrinsic to the Kir6.2 subunit and which are conferred by the SUR1 subunit. Neither has it been possible to determine whether drugs interact directly with Kir6.2, or if they bind to SUR to mediate changes in K-ATP channel activity.

The inventors have made the surprising discovery that deletion of amino acids from the C-terminus of Kir6.2 can enable independent expression of Kir6.2 in both *Xenopus* oocytes and mammalian cells. This suggests that the presence of the C-terminus either prevents insertion of Kir6.2 into the plasma membrane or inhibits channel activity. The inventors have deleted the last 26 (KIR6.2ΔC26) or 36 (Kir6.2ΔC36) amino acids of Kir6.2. C-terminal deletions of mouse Kir6.2 (Genbank Accession No.D50581) were made by introduction of a stop codon at the appropriate residues by site-directed mutagensis in accordance with standard techniques. Synthesis of mRNA encoding wild-type and the deletion variants and SUR1 were carried out as previously described and in accordance with well-known techniques (Gribble, F.M.et al., 1997 J. Physiol. 498.1, p87-98).

The inventors observed that Kir6.2ΔC26 currents have a similar single-channel conductance (69 ± 2 pS, n=5) to that of Kir6.2ΔC26/SUR1 (73 ± 2 pS, n=5), wtKir6.2/SUR1 (70-76 pS) or native (50-80 pS) K-ATP currents, which indicates that deletion of the C-terminus does not alter the single-channel conductance (see figure 5). The deletion mutants maintain sensitivity to ATP (see figure 6).

The ability of Kir6.2 with a deleted C-terminus to express independently means that it is now possible to examine whether drugs (and other substances) interact directly with Kir6.2. Such deletion mutants therefore have important utility as a drug screen. It is useful to know which subunit (Kir or SUR) of a K-ATP channel a drug interacts with. This is because Kir6.2 serves as the pore-forming subunit in a number of tissues, including pancreatic β-cells, heart, skeletal muscle, brain and, possibly, some smooth muscles. By contrast, the SUR subunit varies between tissues. The β-cell channel contains SUR1, as do some brain K-ATP channels. The heart and possibly skeletal muscle channels contains SUR2A, smooth muscle contains SUR2B, and possibly SUR2C. Other splice variants also exist but their tissue distribution has not been carefully mapped. Clearly, it is important to know with which subunit a given drug interacts with in order to target it to a particular tissue (or not, as the case may be). Knowledge of how the drug interacts (ie with which subunit) also allows one to rule out the possibility of cross-reaction with K-ATP channels in different tissues, since it is quicker to exclude/include such cross-reactivity at the molecular

level then test the tissue itself under a myriad range of conditions.

The inventors have also found that deletion of just five residues from C-terminus does not result in modified expression as explained above. Further that deletion of 42 residues from the C-terminus prevents both independent expression and also co-expression with SUR1.

In light of the above, according to one aspect of the present invention there is provided a nucleic acid molecule which has a nucleotide sequence encoding a variant form of an ATP-sensitive K-channel protein which is without part or all of the C-terminal which appears in the wild-type protein and which even when not complexed to a SUR (eg SUR1) has one or more of the following characteristics of an ATP-sensitive K-channel: single-channel conductance; sensitivity to ATP resulting in channel blockage; and channel refreshment (see WO97/18308) following removal of MgATP. Other characteristics of an ATP-sensitive K-channels may be absent eg sensitivity to diazoxide and/or tolbutamide, activation by MgADP or diazoxide and blockage by tolbutamide. Co-expression of a variant form of an ATP-sensitive K-channel protein as stated above, with a SUR (eg SUR1) may or may not confer these properties (drug and MgADP sensitivity) on the variant form.

The variant protein may comprise an amino acid sequence without one or more of the C-terminal amino acid residues shown in Fig.1 and Fig.7. The wild-type protein may include an amino acid sequence as shown in Fig.1 or Fig.2. The variant protein may include an amino acid sequence as shown in Fig.4 (a) or (b). The variant protein may have fewer than 26 amino acid residues deleted from the wild-type C-terminal. The variant protein may have greater than 26 amino acid residues deleted from the wild-type C-terminal; it may have eg 36 amino acid residues deleted or greater than 36 amino acid residues deleted. One skilled in the art will be able to readily ascertain the minimum number of amino acid residues to delete from a wild-type ATP-sensitive K-channel protein sequence in order to obtain a variant form which despite not being complexed to a SUR (eg SUR1) has ATP-sensitive K-channel activity.

The nucleotide coding sequence may be that shown in Fig.4(a) or (b) or it may be a mutant, variant, derivative, allele or fragment of the sequences shown. The sequence may differ from those shown by a change which is one or more of addition, insertion, deletion and substitution of one or more nucleotides of the sequences shown. Changes to a nucleotide sequence as shown may result in an amino acid change at the protein level, or not, as determined by the genetic code.

Thus, nucleic acid according to the present invention may include a nucleotide sequence different from those sequences shown in Fig.4 (a) or (b), which yet still encodes an ATP-sensitive K-channel protein variant as stated above.

Thus, nucleic acid according to the present invention may include a nucleotide sequence different from the sequences shown in Fig.4, yet which encodes a protein with the same amino acid sequence as shown.

On the other hand, the encoded protein may comprise an amino acid sequence which differs by one or more amino acid residues from the amino acid sequences shown in Fig.4.

Nucleic acid encoding an ATP-sensitive K-channel protein variant as stated above, such a protein being an amino acid sequence mutant, variant, derivative or allele of an amino acid sequence as shown in Fig.4 is further provided by the present invention. Such proteins are discussed below.

In relation to the above it is to be stated that variants as stated above of an ATP-sensitive K-channel protein such as Kir6.2 may couple to different kinds of SUR. Thus Kir6.2 variants may be complexed with SUR1 or SUR2 or their splice variants or to other SUR family members and to other members of the ABC transporter family eg the cystic fibrosis transmembrane conductance regulator (CFTCR) or multi-drug resistance related protein (MDRRP). Thus references to SUR herein may be construed as being a reference to any of the SUR family members or members of the ABC transporter family. Thus references to Kir6.2/SUR or references to SUR should be construed as covering all forms of SUR including SUR1, SUR2 and splice variants.

Nucleic acid encoding such a protein may show 50% or greater homology with a nucleotide coding sequence as shown in Fig.4 and/or greater than about 60% homology, and/or greater than about 70% homology, and/or greater than about 80% homology, and/or greater than about 95% homology.

Generally, nucleic acid according to the present invention is provided as an isolate, in isolated and/or purified form, or free or substantially free of material with which it is naturally associated, such as free or substantially free of nucleic acid flanking the gene in the human genome, except possibly one or more regulatory sequence(s) for expression. Nucleic acid may be wholly or partially synthetic and may include genomic DNA, cDNA or RNA.

The coding sequences of Fig.4 is a DNA sequence. For the

RNA equivalent, T is substituted with U.

Nucleic acid may be provided as part of a replicable vector. Therefore also provided by the present invention are vectors including nucleic acid as set out above, particularly any expression vector from which the encoded protein can be expressed under appropriate conditions. Also provided are host cells containing any such vectors or nucleic acids. Host cells may be Xenopus oocytes. An expression vector in this context is a nucleic acid molecule including nucleic acid encoding a protein of interest and appropriate regulatory sequences for expression of the protein, in an *in vitro* expression system, e.g. reticulocyte lysate, or *in vivo*, e.g. in eukaryotic cells such as COS or CHO cells or in prokaryotic cells such as *E. coli*. This is discussed further below.

A further aspect of the present invention provides a polypeptide which comprises part or all a variant form of an ATP-sensitive K-channel protein which is without part or all of the C-terminal which appears in the wild-type protein and which even when not complexed to a SUR (eg SUR1) has one or more of the following characteristics of an ATP-sensitive K-channel: single-channel conductance; sensitivity to ATP resulting in channel blockage; and channel refreshment (see WO97/18308) following removal of MgATP. Other characteristics of an ATP-sensitive K-channels may be absent eg sensitivity to diazoxide and/or tolbutamide, activation by MgADP or diazoxide and blockage by tolbutamide. Co-expression of a variant form of an ATP-sensitive K-channel protein as stated above, with a SUR (eg SUR1) may or may not confer these properties (drug and MgADP sensitivity) on the variant form.

The wild-type protein may include an amino acid sequence as shown in Fig.1 or Fig.2. The variant protein may include an amino acid sequence as shown in Fig.4 (a) or (b). The variant protein may have fewer than 26 amino acid residues deleted from the wild-type C-terminal. The variant protein may have greater than 26 amino acid residues deleted from the wild-type C-terminal; it may have eg 36 amino acid residues deleted or greater than 36 amino acid residues deleted. One skilled in the art will be able to readily ascertain the minimum number of amino acid residues to delete from a wild-type ATP-sensitive K-channel protein sequence in order to obtain a variant form which despite not being complexed to a SUR (eg SUR1) has ATP-sensitive K-channel activity.

The polypeptide may have an amino acid sequence as shown in Fig.4. A polypeptide may be in isolated and/or purified form, free or substantially free of material with which it is naturally associated, such as other polypeptides or such as human polypeptides or (for

example if produced by expression in a prokaryotic cell) lacking in native glycosylation, e.g. unglycosylated.

Polypeptides which are amino acid sequence variants, alleles, derivatives, mutants or fragments in relation to a polypeptide as stated above are also provided by the present invention. A polypeptide which is a variant, allele, derivative, mutant or fragment may have an amino acid sequence which differs from that given in Fig.4 by one or more of addition, substitution, deletion and insertion of one or more amino acids. Variant polypeptides as set out above may in addition to the features stated, have immunological cross-reactivity with an antibody reactive the polypeptide for which the sequence is given in Fig.4.

A polypeptide which is an amino acid sequence variant, allele, derivative, mutant or fragment of the amino acid sequence shown in Fig.4 may comprise an amino acid sequence which shares greater than about 35% sequence identity with the sequence shown, greater than about 40%, greater than about 50%, greater than about 60%, greater than about 70%, greater than about 80%, greater than about 90% or greater than about 95%. The sequence may share greater than about 60% similarity, greater than about 70% similarity, greater than about 80% similarity or greater than about 90% or greater than about 95% similarity with an amino acid sequence as shown in Fig.4. Particular amino acid sequence variants may differ from that shown in Fig.4 by insertion, addition, substitution or deletion of 1 amino acid, 2, 3, 4, 5-10, 10-20 20-30, 30-50, 50-100, 100-150, or more than 150 amino acids.

A polypeptide according to the present invention may be isolated and/or purified (e.g. using an antibody), for instance after production by expression from encoding nucleic acid (for which see below). Polypeptides according to the present invention may also be generated wholly or partly by chemical synthesis. The isolated and/or purified polypeptide may be used in formulation of a composition, which may include at least one additional component, for example a pharmaceutical composition including a pharmaceutically acceptable excipient, vehicle or carrier. A composition including a polypeptide according to the invention may be used in prophylactic and/or therapeutic treatment as discussed below.

A polypeptide, peptide fragment, allele, mutant or variant according to the present invention may be used as an immunogen or otherwise in obtaining specific antibodies. Antibodies are useful in purification and other manipulation of polypeptides and peptides, diagnostic screening and therapeutic contexts. This is discussed further below.

Identification of the Kir6.2 C-terminal variants as discussed above allows screening for molecules which affect or modulate its activity or function and which interact directly with an inward rectifier K-channel subunit as opposed to either a SUR or Kir-SUR complex. Such molecules may be useful in a therapeutic (possibly including prophylactic) context.

It is well known that pharmaceutical research leading to the identification of a new drug may involve the screening of very large numbers of candidate substances, both before and even after a lead compound has been found. This is one factor which makes pharmaceutical research very expensive and time-consuming. Means for assisting in the screening process can have considerable commercial importance and utility. Such means for screening for substances potentially useful in treating or preventing a disease associated with abnormal coupling of cellular metabolism and electro-physiological activity is provided by polypeptides according to the present invention. Substances identified as modulators of the polypeptide represent an advance in the fight against such diseases since they provide basis for design and investigation of therapeutics for *in vivo* use.

The skilled person will be readily able to devise screening systems utilising eg polypeptides as hereby provided. For example, a method of screening for a substance which modulates activity (in either an upwards or downwards manner) of a polypeptide variant as stated above may include contacting one or more test substances with such a polypeptide variant in a suitable reaction medium, testing the activity of the polypeptide in the presence of the test substance or substances and comparing that activity with the activity of the polypeptide in comparable reaction medium without the test substance or substances. A difference in activity between the treated and untreated polypeptides is indicative of a modulating effect, be it positive or negative, of the relevant test substance or substances. Thus one may for example screen for modulating substances by examining changes in the properties (eg electrophysiological or pharmacological) of Kir6.2 C-terminal deletion variants. The screening system may involve comparing how a native K-ATP channel (comprising eg Kir6.2 complexed to SUR1) and how a polypeptide variant as herein provided (eg Kir6.2CΔ26, Kir6.2CΔ36) respond to a test substance.

Thus the effects of the test substance on a Kir6.2/SUR complex may first be established. The measurement will be of any factor indicative of change in the current flowing through the K-ATP channel (Kir6.2 subunit). Thus one may measure membrane current, or the change in membrane potential induced by the membrane current or the change in cytosotic calcium which is linked to the change

in membrane channel. Those of skill in the art will be aware of other factors indicative of current change which may be measured. Having measured the effects of the test substance on a Kir6.2/SUR complex one may similarly measure the effects of the test substance on a Kir6.2 variant eg a deletion mutant as hereby provided and which surprisingly despite not being complexed to SUR1 function as a K-ATP channel.

The results will allow the skilled person to determine the K-ATP subunit with which the drug is interacting. Mutations/alterations may then be introduced into the subunit sequences to establish the binding motif for the drug. Knowledge of the binding motif will allow for accurate drug screening and design.

Thus identification of the Kir6.2, C-terminal deletion variants enables the design of simple and specific screening systems for testing the efficacy of drugs on channel activity. Screens which are based upon the use of the entire Kir 6.2 channel protein or nucleotide sequence, or just a fragment of these can be envisaged. For instance, a useful screen could be derived from a fragment of the Kir6.2, C-terminal deletion variants or functional equivalents thereof. The variant may include the binding site of the Kir6.2 channel to a SUR.

For example, a nucleotide sequence as provided (eg as shown in Fig.4) may be transfected (permanently or transiently) into a cell line such as a mammalian cell line, and electrophysiological or binding studies implemented to establish the potency of drug efficacy or drug binding (eg by flux studies, electrophysiological studies, etc.).

Alternatively, binding studies may be carried out *in vitro*, for example by detecting binding a polypeptide as provided (eg as shown in Fig.4) and a drug under test. The studies may be carried out using conventional techniques. For instance, one component of the prospective binding pair may be immobilised on a support means and the other administered to said support. Binding may be detected using conventional techniques using for example a labelling means such as a labelled antibody.

As used herein the expression "functional equivalent" means a peptide which although different in amino acid structure, has a similar biological effect in the context in which the peptide is used.

Novel drugs identified by the screening methods of the invention form yet a further aspect of the invention.

Combinatorial library technology provides an efficient way of testing a potentially vast number of different

substances for ability to modulate activity of a polypeptide.

Prior to, or as well, as being screened for modulation of activity, test substances may be screened for ability to interact with the polypeptide, e.g. in a yeast two-hybrid system (which requires that both the polypeptide and the test substance can be expressed in yeast from the encoding nucleic acid). This may be used as a coarse screen prior to testing a substance for actual ability to modulate activity of variant polypeptides as provided.

Following identification of a substance which modulates or affects polypeptide activity, the substance may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals.

Thus, the present invention extends in various aspects not only to a substance identified using materials and methods in accordance with what is disclosed herein, but also to a pharmaceutical composition, medicament, drug or other composition comprising such a substance, a method comprising administration of such a composition to a patient, use of such a substance in manufacture of a composition for administration, and a method of making a pharmaceutical composition comprising admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients. Diseases which may benefit from use of the various approaches described herein are listed below.

A substance identified as a modulator of variant polypeptide function may be peptide or non-peptide in nature. Non-peptide "small molecules" are often preferred for many *in vivo* pharmaceutical uses. Accordingly, a mimetic or mimic of the substance (particularly if a peptide) may be designed for pharmaceutical use.

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, e.g. peptides are not well suited as active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing may be used to avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property.

Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled to according its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

Mimetics of substances identified as having ability to modulate variant polypeptide activity using a screening method as disclosed herein are included within the scope of the present invention.

A polypeptide, peptide or substance able to modulate activity of a variant polypeptide according to the present invention may be provided in a kit, e.g. sealed in a suitable container which protects its contents from the external environment. Such a kit may include instructions for use.

A convenient way of producing polypeptides is to express nucleic acid encoding it, by use of the nucleic acid in an expression system.

Accordingly, the present invention also encompasses a

method of making a polypeptide (as disclosed), the method including expression from nucleic acid encoding the polypeptide (generally nucleic acid according to the invention). This may conveniently be achieved by growing a host cell in culture, containing such a vector, under appropriate conditions which cause or allow expression of the polypeptide. Polypeptides may also be expressed in *in vitro* systems, such as reticulocyte lysate.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, eukaryotic cells such as mammalian and yeast, and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptides include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, COS cells and many others. A common, preferred bacterial host is *E. coli*. Also of interest are Xenopus oocytes.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Current Protocols in Molecular Biology*, Ausubel *et al.* eds., John Wiley & Sons, 1992.

Thus, a further aspect of the present invention provides a host cell containing nucleic acid as disclosed herein. The nucleic acid of the invention may be integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques. The nucleic acid may be on an extra-chromosomal vector within the cell.

A still further aspect provides a method which includes introducing the nucleic acid into a host cell. The introduction, which may (particularly for *in vitro* introduction) be generally referred to without limitation as "transformation", may employ any available technique. For eukaryotic cells, suitable techniques may include direct injection of mRNA or DNA (transient transfections), calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium

chloride transformation, electroporation and transfection using bacteriophage.

Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones containing nucleic acid of interest, as is well known in the art.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells (which may include cells actually transformed although more likely the cells will be descendants of the transformed cells) under conditions for expression of the gene, so that the encoded polypeptide is produced. If the polypeptide is expressed coupled to an appropriate signal leader peptide it may be secreted from the cell into the culture medium. Following production by expression, a polypeptide may be isolated and/or purified from the host cell and/or culture medium, as the case may be, and subsequently used as desired, e.g. in the formulation of a composition which may include one or more additional components, such as a pharmaceutical composition which includes one or more pharmaceutically acceptable excipients, vehicles or carriers (e.g. see below).

Introduction of nucleic acid may take place *in vivo* by way of gene therapy, as discussed below.

A host cell containing nucleic acid according to the present invention, e.g. as a result of introduction of the nucleic acid into the cell or into an ancestor of the cell and/or genetic alteration of the sequence endogenous to the cell or ancestor (which introduction or alteration may take place *in vivo* or *ex vivo*), may be comprised (e.g. in the soma) within an organism which is an animal, particularly a mammal, which may be human or non-human, such as rabbit, guinea pig, rat, mouse or other rodent, cat, dog, pig, sheep, goat, cattle or horse, or which is a bird, such as a chicken. Genetically modified or transgenic animals or birds comprising such a cell are also provided as further aspects of the present invention.

This may have a therapeutic aim. The presence of a mutant, allele or variant sequence within cells of an organism, particularly when in place of a homologous endogenous sequence, may allow the organism to be used as a model in testing and/or studying the role of the gene or substances which modulate activity of the encoded polypeptide *in vitro* or are otherwise indicated to be of therapeutic potential.

Instead of, or as well, as being used for the production of a polypeptide encoded by a transgene, host cells may be used as a nucleic acid factory to replicate the nucleic acid of interest in order to generate large

amounts of it. Multiple copies of nucleic acid of interest may be made within a cell when coupled to an amplifiable gene such as DHFR. Host cells transformed with nucleic acid of interest, or which are descended from host cells into which nucleic acid was introduced, may be cultured under suitable conditions, e.g. in a fermentor, taken from the culture and subjected to processing to purify the nucleic acid. Following purification, the nucleic acid or one or more fragments thereof may be used as desired, for instance in a diagnostic or prognostic assay as discussed elsewhere herein.

The provision of the new variant polypeptides as described herein enables the production of antibodies able to bind to such polypeptides. Accordingly, a further aspect of the present invention provides an antibody able to bind specifically to a polypeptide as provided herein (eg a polypeptide whose sequence is given in Fig.4). Such an antibody may be specific in the sense of being able to distinguish between the polypeptide it is able to bind and other human polypeptides for which it has no or substantially no binding affinity (e.g. a binding affinity of about 1000x worse). Specific antibodies bind an epitope on the molecule which is either not present, or is not accessible, on other molecules. Antibodies are also useful in purifying the polypeptide or polypeptides to which they bind, e.g. following production by recombinant expression from encoding nucleic acid.

Preferred antibodies according to the invention are isolated, in the sense of being free from contaminants such as antibodies able to bind other polypeptides and/or free of serum components. Monoclonal antibodies are preferred for some purposes, though polyclonal antibodies are within the scope of the present invention.

Antibodies may be obtained using techniques which are standard in the art. Methods of producing antibodies include immunising a mammal (e.g. mouse, rat, rabbit, horse, goat, sheep or monkey) with the protein or a fragment thereof. Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and screened, preferably using binding of antibody to antigen of interest. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage *et al.*, 1992, Nature 357: 80-82). Isolation of antibodies and/or antibody-producing cells from an animal may be accompanied by a step of sacrificing the animal.

As an alternative or supplement to immunising a mammal with a peptide, an antibody specific for a protein may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which

display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047. The library may be naive, that is constructed from sequences obtained from an organism which has not been immunised with any of the proteins (or fragments), or may be one constructed using sequences obtained from an organism which has been exposed to the antigen of interest.

Antibodies according to the present invention may be modified in a number of ways. Indeed the term "antibody" should be construed as covering any binding substance having a binding domain with the required specificity. Thus the invention covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including synthetic molecules and molecules whose shape mimics that of an antibody enabling it to bind an antigen or epitope.

Example antibody fragments, capable of binding an antigen or other binding partner are the Fab fragment consisting of the VL, VH, Cl and CH1 domains; the Fd fragment consisting of the VH and CH1 domains; the Fv fragment consisting of the VL and VH domains of a single arm of an antibody; the dAb fragment which consists of a VH domain; isolated CDR regions and F(ab')2 fragments, a bivalent fragment including two Fab fragments linked by a disulphide bridge at the hinge region. Single chain Fv fragments are also included.

A hybridoma producing a monoclonal antibody according to the present invention may be subject to genetic mutation or other changes. It will further be understood by those skilled in the art that a monoclonal antibody can be subjected to the techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP184187A, GB 2188638A or EP-A-0239400. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023.

Hybridomas capable of producing antibody with desired binding characteristics are within the scope of the present invention, as are host cells, eukaryotic or prokaryotic, containing nucleic acid encoding antibodies (including antibody fragments) and capable of their expression. The invention also provides methods of production of the antibodies including growing a cell capable of producing the antibody under conditions in which the antibody is produced, and preferably secreted.

The reactivities of antibodies on a sample may be

determined by any appropriate means. Tagging with individual reporter molecules is one possibility. The reporter molecules may directly or indirectly generate detectable, and preferably measurable, signals. The linkage of reporter molecules may be directly or indirectly, covalently, e.g. via a peptide bond or non-covalently. Linkage via a peptide bond may be as a result of recombinant expression of a gene fusion encoding antibody and reporter molecule.

One favoured mode is by covalent linkage of each antibody with an individual fluorochrome, phosphor or laser dye with spectrally isolated absorption or emission characteristics. Suitable fluorochromes include fluorescein, rhodamine, phycoerythrin and Texas Red. Suitable chromogenic dyes include diaminobenzidine.

Other reporters include macromolecular colloidal particles or particulate material such as latex beads that are coloured, magnetic or paramagnetic, and biologically or chemically active agents that can directly or indirectly cause detectable signals to be visually observed, electronically detected or otherwise recorded. These molecules may be enzymes which catalyse reactions that develop or change colours or cause changes in electrical properties, for example. They may be molecularly excitable, such that electronic transitions between energy states result in characteristic spectral absorptions or emissions. They may include chemical entities used in conjunction with biosensors. Biotin/avidin or biotin/streptavidin and alkaline phosphatase detection systems may be employed.

The mode of determining binding is not a feature of the present invention and those skilled in the art are able to choose a suitable mode according to their preference and general knowledge.

Antibodies according to the present invention may be used in screening for the presence of a polypeptide, for example in a test sample containing cells or cell lysate as discussed, and may be used in purifying and/or isolating a polypeptide according to the present invention, for instance following production of the polypeptide by expression from encoding nucleic acid therefor.

An antibody may be provided in a kit, which may include instructions for use of the antibody, e.g. in determining the presence of a particular substance in a test sample. One or more other reagents may be included, such as labelling molecules, buffer solutions, elutants and so on. Reagents may be provided within containers which protect them from the external environment, such as a sealed vial.

Deletion of up to 36 amino acids of the C-terminus of

Kir6.2 enables independent functional expression of Kir6.2. The inventors have examined two mutants: Kir6.2ΔC26 in which 26 amino acids have been deleted and Kir6.2ΔC36 in which 36 amino acids were deleted (see above). Both give large ATP-sensitive K-currents when expressed alone in either *Xenopus* oocytes or mammalian cells. Tolbutamide and diazoxide were without effect on Kir6.2ΔC26 or Kir6.2ΔC36 currents at concentrations which markedly alter wild-type (wt) Kir6.2/SUR1 currents. This indicates that Kir6.2 is not intrinsically sensitive to either of these drugs. Coexpression of Kir6.2ΔC26 with SUR1 restored the efficacy of diazoxide to that found for wild-type K-ATP currents. It also partially restored the effects of tolbutamide (~80%). These data confirm that SUR1 endows the K-ATP channel with sensitivity to sulphonylureas and diazoxide and indicate that the Kir6.2ΔC26 is still capable of coupling to SUR1. Kir6.2ΔC36 also remains capable of coupling to SUR1 (see Tucker S. J. et al., (1997) Nature Vol 387 p179-183). Therefore the last 36 residues of the wild type protein appear not to be critical for binding to SUR1.

In accordance with the teachings of this specification alternative mutant forms of Kir6.2 may be constructed and tested to identify inter alia the site on Kir6.2 with which SUR1 interacts to mediate drug sensitivity. This will enable the design of new drugs targeted to this site and equivalent sites on other Kirs which mimic the effects of either K-channel openers or sulphonylureas. Binding of some K-channel openers (eg diazoxide) to a SUR (such as SUR1) results in channel opening, whereas binding of the same sulphonylureas to a SUR such as SUR2 does not. Likewise other K channel openers interact with Kir6.2/SUR2A channels, but not with Kir6.2/SUR1 (or SUR2B). Both these effects are transduced via interaction of the SUR with the C-terminus of eg Kir6.2. A drug which mimics the changes in eg Kir6.2 which occur when SUR1 mediates its effects (up or down regulation) should open or close the channel directly. Thus the present disclosure provides for the targetting of molecules of potential value as drugs directly to eg the Kir/SUR (eg Kir6.2/SUR1) interaction site which may be identified by utilisation of the teachings herein. Drugs may be small molecules other than peptides/polypeptides or they may be peptides/polypeptides. Equivalent interaction sites will exist on other Kirs eg Kir6.1 and will be involved in the coupling of the protein to sulphonylurea receptors. Therefore one may design drugs which are common to Kir6.2 and Kir6.1, or which discriminate between them.

In broad outline, one may use substances comprising the amino acid residues identified as being involved in the Kir (eg Kir6.2) interaction site to screen for (see above for general comments on screening systems) binding partners which interact with those residues. Binding

partners may then be investigated for their biochemical/physiological effects on the Kir (eg Kir6.2), particularly binding partners which compete with and/or mimic the effects of SUR (eg SUR1) in order to explore their value as drugs. A lead compound can be used to design mimetics as mentioned earlier.

In light of the above, the present invention provides a nucleic acid molecule which has a nucleotide sequence encoding part or all of a region of an ATP-sensitive K-channel protein which interacts with a SUR (eg SUR1). The region may comprise a sequence of amino acids from the region around amino acids 1 to 355 of Kir6.2 shown in Fig.2. Alternatively, the region may comprise a sequence of amino acids from a region of a Kir protein/polypeptide which is substantially homologous to this sequence.

Also provided are functionally equivalent and/or related variants as mentioned above (in relation to Kir.6.2 deletion variants) eg mutants, variants derivatives, alleles or fragments of the sequences shown; the nucleotide sequences may be employed as generally set forth above eg in vectors, host cells, expression systems.

The invention also provides oligopeptides/polypeptides forming part or all of a region of an ATP-sensitive K-channel protein which interacts with a SUR (eg SUR1).

Oligopeptides/polypeptides which are amino acid sequence variants, alleles, derivatives, mutants or fragments of oligopeptides/polypeptides as stated above, are also provided. The general nature of these alternative forms are as set out above (in relation to Kir.6.2 deletion variants).

As stated above the identification of the site on Kir6.2 with which SUR1 interacts to mediate drug sensitivity now enables the design of new drugs targeted to this site, which mimic the effects of either K-channel openers or sulphonylureas.

This renders it possible to design new drugs targeted to around this site which mimic the effects of the known K-channel openers or sulphonylureas. Having identified the area of a K-ATP channel (eg Kir6.2) apparently critical to the interaction with a SUR, those involved in the field of drug design would be readily able to design new drugs specifically targeted to the K-ATP channel)SUR interaction site.

The provision of new oligopeptides/polypeptides and nucleic acid molecules provides for the applications of these substances all generally as described earlier in relation to the Kir 6.2 deletion variants eg screening

methods, products found by use of the screening methods, production of substances with antibody binding domains and such substances themselves, medicaments, kits etc.

The imidazolines are a group of drugs that contain an imidazoline nucleus. They include some compounds that either block or activate α-adrenoreceptors. Several members of this group act as potent stimulators of insulin secretion (for review, see Chan,S.L.F. 1993, Clinical Science 85, p671-677 and Chan,S.L.F. 1991 Eur. J. Pharmacol. 204,41-48). There is good evidence that their insulinotropic effects result from inhibition of ATP-sensitive K-channels (K-ATP channels) in the β-cell plasma membrane (Dunne, M.J.. et al. 1995 Ann. NY Acad. Sci., 763 p242-261). The activity of K-ATP channels sets the β-cell resting potential and their inhibition by imidazolines leads to membrane depolarization, activation of Ca^{2+}-dependent electrical activity and a rise in $[Ca^{2+}]_i$ which triggers insulin release (Ashcroft, F.M. & Rorsman, P. 1989 Prog. Biophys. Molec. Biol. 54 p 87-143). Single-channel studies have shown that imidazolines inhibit K-ATP channels via a decrease in the channel open probability, leaving the single-channel conductance unaffected (Dunne, M.J. et al., 1995 supra.).

Imidazoline receptors have been broadly classified into two types: type I1 has nanomolar affinity for clonidine analogues and type I2 has micromolar affinity for clonidine (Michel, M.C. and Ernsberger, P. 1992 TIPS 13, p369-370). The imidazoline receptor of the pancreatic β-cell, however, has a unique pharmacology and is believed to correspond to a third type of imidazoline receptor (Olmos, G. et al. 1994 Eir.J. Pharmacol. 262, p41-48). The identity of this receptor is unknown and has been the subject of much research. Results to date have done nothing to indicate or establish whether the imidazoline receptor corresponds to the either of the subunits (Kir6.2 or SUR1) of the β-cell K-ATP channel itself, or whether it resides on a separate protein which interacts with the K-ATP channel.

The present inventors now have results which, for the first time, indicate that surprisingly the site at which imidazoline drugs bind to mediate K-ATP channel inhibition lies on Kir6.2 and that this site lies on the intracellular side of the membrane. The evidence in support of this idea is summarised below.

(a) Co-expression of Kir6.2 and SUR1 in Xenopus oocytes gives rise to large ATP-sensitive K-currents. When imidazolines such as phentolamine and efaroxan are added to the intracellular membrane surface of such patches they act as potent blockers of K-ATP currents. For example, 10μM phentolamine produces complete block of K-ATP currents. This argues that the binding site for imidazoline drugs lies either on the Kir6.2 or on the

SUR1 subunit of the K-ATP channel.

(b) The problem of which K-ATP channel subunit contains the phentolamine site has been difficult to address because, unlike most other Kir channels, Kir6.2 does not express functional currents alone, but only when coexpressed with SUR1. The inventors have discovered that modified forms of Kir6.2 which are without the last 26 (Kir6.2ΔC26) or 36 (Kir6.2ΔC36) amino acids of the C-terminus, are electrophysiologically functional absent coexpression with SUR1. The C-terminally-deleted Kir6.2 currents (Kir6.2ΔC26, Kir6.2ΔC36) were blocked by phentolamine as potently as wtKir6.2/SUR1 currents (see eg Fig.2 of Proks, P., et al. 1997, Proc. Natl. Acad. Sci., USA Vol 94 p11716 to 11720). This indicates that the binding site for imidazoline drugs lies on Kir6.2.

(c) The inventor's results also indicate that the binding site for imidazolines is located on the cytosolic side of the transmembrane Kir6.2 protein (see Fig.7). This is because the imidazoline phentolamine was without effect on wtKir6.2/SUR1 currents expressed in *Xenopus* oocytes when applied to the external membrane surface, yet caused pronounced inhibition of K-ATP currents when applied to the intracellular membrane surface of excised membrane patches. The effects of phentolamine on native K-ATP currents in β-cells are consistent with the idea that the drug must reach its target site by traversing the membrane. Thus external application of the drug produces a slow and irreversible block of whole-cell K-ATP currents, whereas when it is added to the intracellular solution, phentolamine causes a rapid and reversible block of single-channel K-ATP currents (Dunne,M.J. 1991 Br.J.Pharmacol 103, p1847-1850).

(d) The inventors have localised the imidazoline site more precisely. Firstly, as it appears to reside on the intracellular side of the membrane, it must involve amino acids in the N and/or C-terminal domains or within the inner mouth of the pore region (which may include the putative transmembrane domain) of the Kir6.2 (see Fig.7). Secondly, deletion of the last 36 residues of Kir6.2 (355-end) does not affect phentolamine sensitivity and it thus is unlikely to form part of the drug-binding site. Thirdly, the related Kir channel, Kir1.1, which expresses independently, is insensitive to phentolamine and efaroxan. Thus the binding site (and/or sites involved in transducing drug-binding into closure of the pore) must include residues which are different in Kir6.2 and Kir1.1. Also mutation K185Q is phentolamine sensitive, so this residue is unlikely to represent a binding site.

Thus the present invention provides nucleotide and polypeptide sequences for a Kir channel binding site which is able to bind molecules containing an imidazoline nucleus and which binding site is at a polypeptide

location which in the membrane lies intracellularly.

The Kir channel may be Kir6.2 and in which case the binding site may comprise some residues not common between Kir6.2 and Kir1.1a (see Figure 7 and Figure 8). However some residues of the binding site, may be common to Kir6.2 and Kir1.1a.

The identification of a drug binding site such as the binding site for molecules comprising an imidazoline nucleus allows the design of new drugs which interact with the site in accordance with ordinary techniques used by those in the field of drug design and as generally discussed earlier herein.

Molecules of therapeutic value in relation to the treatment of a disease selected from diabetes mellitus including non-insulin-dependent diabetes; maturity-onset-diabetes of the young and insulin-dependent diabetes; diseases which result in increased insulin secretion such as persistent hyperinsulinaemia of infancy; hypertension and hypotension; cerebral ischaemia; stroke; cerebral vasospasm; peripheral arterial disease; vascular smooth muscle disease; angina pectoris; long QT syndrome; vasospastic angina; cardiac dysrhythmia; ischaemia induced cardiac arrhythmias; Parkinson's Disease; diseases associated with abnormal cerebellar function (eg ataxia) asthma; diseases of intestinal smooth muscle, including irritable bowel syndrome, urinary incontinence; male alopecia; certain skeletal muscle myopathies, such as those associated with membrane depolarisation or hyperpolarisation or hypopolarisation; appetite (both reduced and excessive); abnormalities and diseases of uterine smooth muscle, including premature labour; diseases involving abnormal nerve conduction; inappropriate secretion of renin and insensitivity to certain classes of drugs (eg sulphonylureas, K-channel openers etc) caused by inappropriate coupling between Kir6.2 and the drug-binding subunits of the channel complex, may be identified and provided by use of the materials and methods described herein.

The ensuing passages provide experimental evidence supporting the new disclosures discussed above.

The following experiments provide support for the disclosure that Kir6.2 with a C-terminal deletion can express and function electrophysiologically independently of SUR1. The inventors results also show that deletion of 26 and 36 residues does not abolish sensitivity to tolbutamide or diazoxide when the deletion variant is coexpressed with SUR1 (see eg Tucker S. J., et al. 1997 Nature Vol 387 p179 to 183).

The inventors investigated effect of deleting the last 26 (Kir6.2ΔC26) or 36 (Kir6.2ΔC36) amino acids of the C-

terminus of Kir6.2 (see Figures 4, 5 and 6).

Large whole-cell currents can be recorded in response to metabolic inhibition from *Xenopus* oocytes coinjected with mRNAs encoding wild-type (wt) Kir6.2 and SUR1, but not from oocytes injected with wtKir6.2 mRNA alone (Fig.9 C,D). In contrast, detectable currents were recorded from oocytes injected either with Kir6.2ΔC26 or Kir6.2ΔC36 mRNAs. These currents were further enhanced by metabolic inhibition. This suggests that Kir6.2ΔC26 and Kir6.2ΔC36 are not only capable of independent expression but are also intrinsically sensitive to metabolically-induced changes in cytosolic nucleotide levels. The fact that deletion of the C-terminus of Kir6.2 enables it to express independently of SUR1 further suggests that the presence of the C-terminus either prevents insertion of Kir6.2 into the plasma membrane or inhibits channel activity.

Fig. 10 shows single-channel currents and mean single-channel current-voltage relations recorded from inside-out patches excised from oocytes expressing Kir6.2ΔC26 or Kir6.2ΔC26 plus SUR1. The single-channel conductance, measured between -20mV and -80mV, was 69±2 pS (n=4) for Kir6.2ΔC26 and 73±2 pS (n=5) for Kir6.2ΔC26 plus SUR1. Thus, SUR1 does not modify the conductance of the K-ATP channel. The single-channel conductance of Kir6.2ΔC36 was 75±2 pS (n=5). None of these values are significantly different from those of wtKir6.2/SUR1 (70-76 pS) or native K-ATP currents (50-80 pS) which indicates that deletion of the C-terminus does not alter the single-channel conductance.

To examine the nucleotide sensitivity of the mutant currents, the inventors recorded macroscopic currents from giant patches excised from *Xenopus* oocytes expressing wtKir6.2, Kir6.2ΔC26 or Kir6.2ΔC36. The effects of other deletions are disclosed in Tucker S. J., et al. 1997 Nature Vol 387 p179 to 183. A marked increase in current was observed when patches from oocytes expressing either of the C-terminal deletions were excised into nucleotide-free solution (Fig. 11B). This reflects the relief of the blocking action of ATP present in the oocyte cytoplasm. Deletion of 36 (Kir6.2ΔC36), rather than 26 (Kir6.2ΔC26), residues produced larger currents on patch excision. No increase in current on patch excision was observed, however, in wtKir6.2 injected oocytes.

Application of ATP to the inner membrane surface markedly inhibited both Kir6.2ΔC26 and Kir6.2ΔC36 currents (Fig. 11A), with a K_i of 106±4μM (n=7) and 128±5μM (n=6), respectively. The Kir6.2 subunit must therefore possess an intrinsic ATP-inhibitory site. The Hill coefficient (*h*) was 1.2±0.1 (n=7) for Kir6.2ΔC26 and 1.0±0.1 (n=6) for Kir6.2ΔC36. Its value of close to unity indicates

that only a single ATP molecule needs to bind to close the channel. In β-cells, ATP inhibition does not require hydrolysis of the nucleotide. This was also the case for Kir6.2ΔC36 currents as ATP-inhibition was unaffected by Mg^{2+} removal: 100μM ATP blocked the slope conductance by 44±2% (n=9) in the presence of 1.4mM Mg^{2+} and by 42±2% (n=6) in Mg^{2+} free solution. Coexpression of SUR1 with Kir6.2ΔC26 increased the potency of ATP inhibition (Fig. 11 A,D) to that found for wild-type K-ATP channels K_i= 13.4±0.3μM (n=8) for Kir6.2ΔC26/SUR1 and 10-28μM for wtKir6.2/SUR1 currents. Thus, although the primary site at which ATP inhibits the K-ATP channel lies on Kir6.2, the presence of SUR1 can enhance the blocking action of the nucleotide. Likewise Ki for ATP-inhibition of Kir6.2ΔC36/SUR1 currents (24.6±2.4μM, n=5) was lower than that of Kir6.2ΔC36 currents (128μM) see Tucker, S. J., et al. 1997 Nature Vol 387 p179 to 183.

As found for the native and wild-type K-ATP currents both Kir6.2ΔC26 (Fig. 11) and Kir6.2ΔC36 currents ran down with time in excised patches, and were refreshed following exposure to MgATP. This indicates that both these properties are intrinsic to Kir6.2 rather than to SUR1.

Both native K-ATP and wtKir6.2/SUR1 currents are potential by MgADP and diazoxide, and blocked by tolbutamide. In contrast, MgADP blocked Kir6.2ΔC26 currents (Fig. 11D). This result is not unexpected since the NBDs of SUR1 confer MgADP activation on the K-ATP channel and in the absence of this activation an inhibitory effect of MgADP is unmasked. It further suggests that, like ATP, the inhibitory effect of ADP is intrinsic to Kir6.2 Fig. 11C also shows that tolbutamide and diazoxide were without effect on Kir6.2ΔC26 currents at concentrations which markedly alter wtKir6.2/SUR1 currents. Coexpression of Kir6.2ΔC26 with SUR1 restored the efficacy of diazoxide to that found for wild-type currents. It also partially restored the effects of tolbutamide and MgADP. These data confirm that SUR1 endows the K-ATP channel with sensitivity to sulphonylureas, diazoxide and the potentiatory action of MgADP.

To exclude the possibility that Kir6.2ΔC36 (or Kir6.2ΔC26) couples to an endogenous protein in the *Xenopus* oocyte which endows it with ATP-sensitivity, we also examined whether the protein expresses ATP-sensitive currents in mammalian cells. Whole-cell currents recorded from HEK293 cells transfected with Kir6.2ΔC36 increased with time following establishment of the whole-cell configuration when dialysed intracellularly with 0.3mM ATP (Fig. 12). This increase in current reflects the washout of endogenous ATP from the cell as it was not observed when cells were dialysed with 5mM ATP. Similar results were observed for Kir6.2ΔC26 currents (not shown) and for wtKir6.2/SUR1 currents. In general, channels

which require an additional subunit for expression in oocytes (eg Kir3.1, IsK) do not express by themselves in mammalian cells and only express small currents in *Xenopus* oocytes. Thus the results strongly argue that Kir6.2ΔC26 and Kir6.2ΔC36 are capable of independent expression and are intrinsically ATP-sensitive.

The inventors have also shown that neutralisation of the lysine residue at position 185 to glutamine (Kir6.2ΔC26-K185Q) substantially decreased the channel sensitivity to ATP. This data supporting the idea that ATP interacts directly with Kir6.2 to mediate channel inhibition and that lysine 185 is involved in this inhibition either by forming part of the ATP binding site itself or by constituting part of the transduction mechanism by which binding of ATP to a site elsewhere effects channel closure (Tucker S. J. et al. 1997 supra eg Fig 4). The inventor has also conclusively shown that Kir6.2ΔC26 and Kir6.2ΔC36 can couple to SUR1 and have normal drug sensitivity (Tucker S. J. et al. 1997 supra; eg Fig 2).

In broad conclusion, the ability of the C-terminal deleted Kir6.2 to express independently has allowed the inventors to investigate which properties of the K-ATP channels are intrinsic to the Kir6.2 subunit and which are conferred by the SUR1 subunit. The results provide evidence that Kir6.2 acts as the pore of the K-ATP channel complex and confirm that SUR1 endows Kir6.2 with sensitivity to sulphonylureas, diazoxide and MgADP. Importantly, they also indicate that the primary site at which ATP interacts to mediate channel inhibition is located on Kir6.2 and not on SUR1. However, although the inhibitory ATP-binding site lies on Kir6.2, it appears that SUR1 enhances the sensitivity of Kir6.2 to ATP. The demonstration that the inhibitory ATP-binding site lies on Kir6.2 allows identification of the exact residues involved in ATP binding and of the mechanism by which ATP-binding leads to inhibition of channel activity.

Methods

Molecular Biology: C-terminal deletions of mouse Kir6.2 (Genbank D50581) were made by introduction of a stop codon at the appropriate residues by site-directed mutagenesis. Synthesis of mRNA encoding wild-type and mutant mouse Kir6.2 and wild-type rat SUR1 (Genbank L40624) was carried out as previously described (Gribble, F.M., et al., J Physiol. 498.1, 87-98 (1997).

Electrophysiology: Xenopus oocytes were defolliculated and coinjected with 10ng each of mRNAs encoding wtKir6.2, Kir6.2ΔC26 or Kir6.2ΔC36, with or without SUR1. The final injection volume ~50 nl/oocyte. Isolated oocytes were maintained in tissue culture (Gribble, F.M., et al., 1997 supra) and studied 1-4 days after injection. Whole-cell currents were measured at 18-24°C using a standard 2-electrode voltage-clamp in (mM): 90 KCl, 1 $MgCl_2$, 1.8

$CaCl_2$, 5 HEPES (pH 7.4 with KOH). The holding potential was -10mV. Currents were filtered at 1kHz, digitised at 4kHz and measured 280-295 ms after the start of the voltage pulse. Macroscopic (or single-channel) currents were recorded from giant (or normal) excised inside-out patches at a holding potential of 0mV and at 20-24°C (Hilgemann, D.W. et al., Nature 352, 715-718 (1991). The pipette solution contained (mM): 140 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 10 HEPES (pH 7.4 with KOH) and the internal (bath) solution contained (mM): 110 KCl, 1.44 $MgCl_2$, 30 KOH, 10 EGTA, 10 HEPES (pH 7.2 with KOH) plus nucleotides as indicated. The Mg-free solution contained (in mM): 110 KCl, 30 KOH, 2.6 $CaCl_2$, 10 EDTA, 10 HEPES (pH 7.2 with KOH). Macroscopic currents were recorded in response to 5 s voltage ramps from -110mV to +100mV with an EPC7 amplifier (List Electronik, Darmstadt, Germany), filtered at 0.2kHz and sampled at 0.5kHz. The slope conductance was measured by fitting a straight line to the data between -20mV and -100mV: the average of 5 consecutive ramps was calculated in each solution. Single-channel currents were filtered at 1kHz and sampled at 3kHz.

HEK293 cells were transiently transfected with the pCDNA3 vector (Invitrogen) containing the coding sequence of wild-type or mutant Kir6.2 using lipofectamine (Sakura, H., et al., FEBS Lett 377, 338-344 (1995). Whole-cell currents were studied 48-72 hr after transfection. The pipette solution contained (mM): 107 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 5 HEPES (pH 7.2 with KOH; total K ~140mM) and either 0.3 or 5mM ATP. The bath solution contained (mM): 40 KCl, 100 NaCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES (pH 7.4). The holding potential was -30mV.

Data analysis: All data is given as mean ±S.E.M. The symbols in the figures indicate the mean and the vertical bars one S.E.M (where this is larger than the symbol). ATP dose-response relationships were fit to the Hill equation: $G/G_c = O + (100 - O / (1 + ([ATP] / K_i)^n))$, where [ATP] is the ATP concentration, K_i is the ATP concentration at which inhibition is half maximal, n is the slope factor (Hill coefficient) and O describes the background current (not blocked by ATP). Statistical significance was tested using Student's t-test.

Also provided are Figures 13 and 14. Fig. 13 of this series shows that deletion of the last 26 amino acids from the C-terminus of Kir6.2 enables the independent expression of Kir6.2 currents. These currents are not blocked by tolbutamide or diazoxide indicating that sensitivity to these drugs is not intrinsic to Kir6.2. Likewise MgADP has no activating effect. Coexpression with SUR1 endows sensitivity to diazoxide, tolbutamide and the stimulatory effects of MgADP.

Fig. 14 of the series shows the deletion of the last 26 amino acids from the C-terminus of Kir6.2 does not affect

the ability of SUR1 to couple to Kir6.2 and endow it with tolbutamide and diazoxide sensitivity (although it slightly reduces the efficacy of MgADP in stimulating the channel). In contrast, deletion of the last 36 amino acids abolishes the ability of SUR1 to couple to Kir6.2 and endow it with tolbutamide and diazoxide sensitivity (and also MgADP activation).

In relation to the imidazoline binding site data the following experimental data is provided by way of support and explanation.

Fig.15 shows that wild-type K-ATP (Kir6.2 + SUR1) currents are strongly blocked by the imidazolines phentolamine and efaroxan when applied to the inner membrane surface of excised inside-out patches whereas phentolamine is ineffective when applied to the outer membrane surface of intact cells, and efaroxan produces only a small block (Fig.16). This suggests that the binding site for imidazolines lies on the inside of the membrane.

To determine whether imidazoline phentolamine interacted with the Kir6.2 or SUR1 subunit of the K-ATP channel, the inventors tested the effect of 10 M phentolamine on Kir6.2ΔC36 currents. This deletion mutant of Kir6.2 is able to express independently of SUR1. As shown in Fig. 17, the drug produced a profound inhibition of the current when added to the inside of the membrane. This indicates that phentolamine interacts directly with Kir6.2.

The inventors also examined the effect of phentolamine and efaroxan on the related Kir channel, Kir1.1a (ROMK1). Neither of these drugs produced significant block of Kir1.1a currents over the negative voltage range (Fig.18). At high concentrations (>30μM), efaroxan produced a voltage-dependent block of outward Kir1.1 currents.

Methods in Relation the Imidazoline data and Figs 15 to 18.

Xenopus oocytes were defolliculated and coinjected with 10ng of mRNA encoding wtKir6.2+SUR1, Kir6.2ΔC36 (ie with 36 amino acids deleted from the C-terminus), or Kir1.1a (as indicated). The final injection volume was ~50nl per oocyte in all cases. Isolated oocytes were maintained in modified Barth's solution containing (in mM): 88 NaCl, 1 KCl, 1.7 $MgSO_4$, 0.47 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 2.4 $NaHCO_3$, 10 HEPES (pH 7.4), supplemented with 100 U/ml penicillin, 100μg/ml streptomycin and 5mM pyruvate. Currents were studied 1-4 days after injection.

In relation to Fig.15 whole-cell currents were measured using a 2-electrode voltage clamp (Geneclamp 500, Axon

Instruments, Foster City, USA). Voltages were applied and currents recorded using a microcomputer with an Axolab interface and pClamp software (Axon Instruments). Currents were filtered at 1kHz and digitised at 4kHz. Current and voltage electrodes were filled with 3M KCl and had resistances of 0.5-2 MΩ. Transmembrane potential was measured differentially between the intracellular electrode and a second bath electrode positioned close to the oocyte on the downstream side, in order to minimise series resistance errors. The bath electrodes consisted of $Ag/AgCl_2$ pellets connected to the bath by agar bridges filled with 3M KCl. Whole-cell currents were measured 280-295 ms after the start of the voltage pulse. Oocytes were continuously perfused with a solution containing (mM): 90 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES (pH 7.4 with KOH) plus drug as indicated, at 18-24°C. Na-azide was prepared as a stock solution (1000x) in water. All drugs were prepared as a 1000x stock solution in DMSO and diluted as required.

In relation to Figs. 17 and 18 macroscopic currents were recorded from giant excised inside-out patches (Hilgemann D.W. et al., 1991 supra), at 20-24°C using 200-400 kΩ electrodes. The pipette solution contained (mM): 140 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 10 HEPES (pH 7.4 with KOH) and the internal (bath) solution contained (mM): 110 KCl, 1.44 $MgCl_2$, 30 KOH, 10 EGTA, 10 HEPES (pH 7.2 with KOH) and nucleotides as indicated. Rapid exchange of solutions was achieved by positioning the patch in the mouth of one of a series of adjacent inflow pipes placed in the bath. Currents were recorded with an EPC7 amplifier (List Electronik, Darmstadt, Germany), filtered at 0.2kHz and sampled at 0.5kHz.

The foregoing text provides evidence that deletion mutants of Kir6.2 work as a functional K-ATP channel when expressed alone in the absence of SUR and puts forward the value of such mutants in screening for drugs which affect K-ATP channel activity and identifying the site at which such drugs interact with the subunits of the channel. Trapp, S., et al., in PNAS Vol 94 pp8872-8877 (1997) provides a further example of how Kir6.2 deletion mutants can be used to demonstrate which subunit a substance (in this example guanine nucleotides) interacts with. It has shown that GTP blocks K-ATP currents by interaction with Kir6.2 and that the potentiatory effects of GTP are endowed by SUR1. A complex interaction of a drug with the K-ATP channel may therefore be clarified by testing the effect on eg Kir6.2ΔC26 (or Kir6.2ΔC36) alone and then on Kir6.2ΔC26 (or Kir6.2ΔC36) coexpressed with SUR.

In a similar fashion the inventors have used Kir6.2ΔC26 (and Kir6.2ΔC36) to demonstrate that the lipid oleoyl CoA activates K-ATP channels by interaction with the Kir6.2 subunit and not SUR1. Relevant results are shown as

Figure 19. This is the first example of a K-ATP channel stimulatory drug being shown to interact directly and specifically with Kir6.2.

The inventors have used Kir6.2ΔC36 as a drug screen to establish effects of known drugs. For example, mefloquine is used for malarial prophylaxis. One side effect of the drug is that it has been reported to raise serum insulin concentrations and reduce plasma glucose levels in healthy adults. The drug blocks cloned wild-type Kir6.2/SUR1 currents when expressed in *Xenopus* oocytes by 80 ± 4% (n=9) (Gribble, F.M., et al 1997 Diabetologia 40 A103). They have now used Kir6.2ΔC36 to show that this effect is mediated principally by interaction with the Kir6.2 subunit.

Xenopus oocytes were injected with mRNA encoding Kir6.2ΔC36. Isolated oocytes were maintained in tissue culture and studied 1-4 days after injection. Macroscopic currents were recorded from giant excised inside-out patches at a holding potential of 0 mV at 20-24 °C. The pipette solution contained (mM): 140 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 10 HEPES (pH 7.4 with KOH) and the internal (bath) solution contained (mM): 110 KCl, 1.44 $MgCl_2$, 30 KOH, 10 EGTA, 10 HEPES (pH 7.2 with KOH). Mefloquine was prepared as 1000x stock solutions in DMSO and added to the internal solution as required. Macroscopic currents were recorded in response to a series of 4 s voltage ramps from -110 to +100 mV. The slope conductance was measured by fitting a straight line to the data between -20 mV and -100 mV: the average of 5 consecutive ramps was calculated in each solution.

Mefloquine (10 μM) blocked Kir6.2ΔC36 currents by 66 ± 6% (n=7). This indicates that mefloquine interacts directly with the Kir6.2 subunit to bring about inhibition. since Kir6.2 serves as the pore for many types of K_{ATP} channel, including those of the brain, this may account for some of the side effects of the drug.

The deletion mutant may thus be used to screen for new drugs which mimic the effects of mefloquine and to aid in the design drugs with the advantageous features of mefloquine but which are without certain undesirable side effects.

Use of Kir6.2 as a submembrane ATP-sensor (a novel bioassay)

The inventors also provide for the use of Kir6.2 as a submembrane ATP-sensor. Adenosine tri-phosphate (ATP) is a uniquely important molecule in the cell. Energy stored within the high-energy bonds which attach the two terminal phosphate groups is used to power almost all biochemical reactions. ATP may also act as a ligand for certain ion channels, and thereby regulate their activity. There has been considerable debate as to

whether ATP is compartmentalized within cells and, in particular, whether the ATP concentration just below the plasma membrane, experienced by membrane proteins, is the same as that of the bulk cytoplasm. This issue has been difficult to address because there is no indicator of cytosolic ATP, such as those which are available for Ca^{2+}, which is capable of resolving the submembrane [ATP] in real time within a single cell. The inventors have shown that truncated forms of Kir6.2 (Kir6.2ΔC26 and Kir6.2ΔC36) can be used to measure the submembrane ATP level.

Ligand-sensitive ion channels have been previously used to monitor the intracellular and extracellular concentrations of neurotransmitters and ions: for example, the Ca-activated K-channel has been used to monitor the submembrane $[Ca^{2+}]_i$. The wild-type K_{ATP} channel cannot be used as a biosensor in this way because in the intact cell the inhibitory effect of ATP is partially masked by an additional stimulatory action of MgADP [Kakei et al., 1986 FEBS Lett. 208 p63 to 66]. The K_{ATP} channel as previously stated is an octamer of two subunits: an inwardly rectifying K-channel subunit, Kir6.2, which forms the channel pore and contains the inhibitory binding site for ATP, and a sulphonylurea receptor, SUR1 or SUR2, which endows Kir6.2 with sensitivity to drugs and the stimulatory effects of MgADP. Although wild-type channels require both subunits for functional channel activity, mutant form of Kir6.2 with e.g. a C-terminal truncation of 26 (Kir6.2ΔC36) or 36 (Kir6.2ΔC36) amino acids is able to express independently. This channel is inhibited by ATP but is not activated by MgADP, making it a potential tool for monitoring [ATP]s.

Currents recorded from cell-attached patches on oocytes injected with Kir6.2ΔC36 were very small, but increased ~50-fold following patch excision (Fig. 20). This increase in conductance was not observed in water-injected oocytes, indicating it results from activation of Kir6.2ΔC36 currents which are inhibited in the cell-attached configuration. Subsequent application of 1 mM ATP to the intracellular membrane surface largely blocked Kir6.2ΔC36 currents, suggesting that [ATP]s might be responsible for channel inhibition in the intact oocyte. Consistent with this idea, large currents were recorded from cell-attached patches on oocytes expressing the ATP-insensitive Kir channel, Kir1.1a, and no change in conductance was observed on patch excision: the mean conductance was 15.0 ± 3.6 nS cell-attached and 15.5 ± 3.9 nS after excision (n=5).

The most critical test of whether the amplitude of Kir6.2ΔC36 currents in the intact cell reflects [ATP]s is to examine the effects of mutant channels with altered ATP-sensitivity, as these should produce markedly different current amplitudes in cell-attached patches yet

yield similar values of [ATP]s. The inventors made three different mutations which altered the channel ATP-sensitivity (Fig. 21). Half-maximal inhibition (K_i) of wild-type Kir6.2ΔC36 currents was produced by 115 ± 6 μM ATP (h=1.0 ± 0.1, n=11), similar to that reported for Kir6.2ΔC26 currents (106 μM). Mutation of the arginine at position 50 to glycine (R50G) reduced the K_i for ATP-inhibition to 3.4 ± 0.7 mM (h = 1.2 ± 0.2; n=6), mutation of isoleucine 167 to methionine (I167M) decreased the K_i to 640 ± 130 μM (h= 1.2 ± 0.1; n=5), and mutation of glutamate 179 to glutamine (E179Q) reduced the K_i to 300 ± 20 μM (h = 1.3 ± 0.1; n=8). The inventors next measured the increase in conductance which occurred on excision of patches from oocytes expressing Kir6.2ΔC36, Kir6.2ΔC26-E179Q, Kir6.2ΔC26-I167M and Kir6.2ΔC26-R50G (Fig. 22). Those channels which showed lower ATP-sensitivity exhibited larger currents in the cell-attached configuration and a smaller increase in conductance on patch excision (Fig. 22). However, the submembrane ATP concentration calculated from each set of data was similar. There was a reciprocal relation between the K_i for current inhibition by ATP and the increase in conductance on patch excision. This constitutes good evidence that the principal cytosolic regulator of Kir6.2ΔC36 activity is [ATP]s.

It is implicit in the use of Kir6.2ΔC36 to measure [ATP]s, that other cytosolic factors have little effect on channel activity or ATP-sensitivity. The results support this idea, because a similar estimate of [ATP]s is obtained for channels with different K_i for ATP-inhibition. However, it remains possible that the sensitivity of the mutant channels to substances which interact with the ATP-binding site might change in parallel with ATP-sensitivity. The inventors therefore examined the sensitivity of Kir6.2ΔC36 currents to nucleotides other than ATP. Fig. 23 shows that ADP inhibited Kir6.2ΔC36 almost as potently as ATP, the K_i for inhibition being 260 ± 22 μM (h=1.1 ± 0.1, n=5). By contrast, AMP had little inhibitory effect (K_i, 9.2 ± 0.5 mM, h=1.1 ± 0.1, n=6) as was also the case for ITP (7.6 ± 2.0 % inhibition with 1 mM IPT, n=6) and the guanine nucleotides GTP and GDP (K_i= ~6 mM for GTP, 10 mM GDP blocked by ~50%; Ref 13). Another nucleotide suggested to regulate K_{ATP} channel activity in response to metabolism is diadenosine polyphosphate (Ap_4A). This molecule blocked Kir6.2ΔC36 currents with a potency similar to that of ATP, 100 μM Ap_4A producing 48 ± 2 % (n=4) inhibition. These results argue that only ATP, Ap_4A and ADP are potent blockers of Kir6.2ΔC36. The concentrations of Ap_4A and ADP in the bulk cytoplasm are believed to lie within the low and intermediate micromolar range, respectively and are much lower than that of ATP. Thus we assume that the activity of Kir6.2ΔC36 in the intact cell is largely determined by the submembrane ATP level.

The mean value of the submembrane ATP concentration is 4.6 ± 0.3 mM (n=29), and that from the slope of the graph in Fig. 22 is 5.7 mM. These values are approximately double that measured from the whole oocyte using a biochemical method (2.3 mM). The difference may be only apparent and reflect exclusion of ATP from certain compartments (such as the yolk platelets) or it may indicate an inverse ATP=gradient between the membrane and bulk cytosol. The inventors favour the former explanation because a similar difference in cytosolic (6 mM) and total (2.5 mM) [ATP] was found in *Rana* oocytes. Whatever the reason, the present results do not support the widely-held view that the submembrane ATP concentration is lower than that of the bulk cytosol.

In intact *Xenopus* oocytes, inhibition of cell metabolism by 3 mM azide leads to an increase in the whole-cell K_{ATP} current. Using the value for resting [ATP]s obtained above, we can estimate the fall in submembrane [ATP] following metabolic inhibition. The mean submembrane [ATP] fell to 1.2 ± 0.1 mM (n=24) when metabolism was inhibited. This value is similar to that measured biochemically for the bulk cytoplasm (1.7 mM).

In conclusion, the results suggest that Kir62.ΔC36 may be used as a tool to monitor the submembrane ATP concentration in real-time in a single cell. Inside-out patches excised from cells expressing Kir6.2ΔC36 might also be used to detect the local release of ATP from purinergic neurones, as described for acetylcholine release using acetylcholine receptor channels. In a similar fashion, inside-out macropatches containing Kir6.2ΔC36 might serve to monitor exocytosis from single secretory cells, as many types of secretory granule contain ATP. Importantly, our data also demonstrate that the submembrane ATP concentration lies in the millimolar, rather than the micromolar, range.

Methods

Molecular Biology: A C-terminal deletion of 26 (Kir6.2ΔC26) or 36 amino acids (Kir6.2ΔC36) of mouse Kir6.2 (Genbank D50581) was made by introduction of a stop codon at the appropriate residue by site-directed mutagenesis. Site-directed mutations in Kir6.2ΔC26 were made using the pALTER vector. Synthesis of mRNA encoding wild-type and mutant mouse Kir6.2 was carried out as previously described [Gribble, F.M., et al 1997 EMBO J. 16, 1145-1152].

Electrophysiology: *Xenopus* oocytes were defolliculated and injected with ~2 ng of mRNA encoding either Kir6.2ΔC36 or mutated Kir6.2ΔC26. The final injection volume was ~50 nl/oocyte. Kir6.2ΔC36 was used in preference to wild-type Kir6.2ΔC26 as it showed greater expression and larger currents in cell-attached patches, which made for a more accurate estimate of ATP

concentration: there was no difference in the ATP-sensitivity of Kir6.2ΔC36 or Kir6.2ΔC26 currents [Tucker, S.J. et al, 1997, Nature 378, 179-183]. Isolated oocytes were maintained in tissue culture and studied 1-4 days after injection. Whole-cell currents were measured at 18-24 °C using a standard 2-electrode voltage-clamp [Gribble, F.M., et al, 1997, Supra] in (mM): 90 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES (pH 7.4 with KOH). The holding potential was -10 mV. Currents were filtered at 1 kHz, digitised at 4 kHz and measured 280-295 ms after the start of the voltage pulse.

Macroscopic currents were recorded from giant inside-out patches at a holding potential of 0 mV and at 18-24 °C [Gribble, F.M., et al, 1997, Supra]. The pipette solution contained (mM): 140 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 10 HEPES (pH 7.4 with KOH) and the internal (bath) solution contained (mM): 110 KCl, 1.4 $MgCl_2$, 30 KOH, 10 EGTA, 10 HEPES (pH 7.2 with KOH) plus nucleotides as indicated. Macroscopic currents were recorded in response to 3 s voltage ramps from -110 mV to +100 mV with an EPC7 amplifier (List Electronik, Darmstadt, Germany), filtered at 0.2 kHz and sampled at 0.5 kHz. The slope conductance was measured by fitting a straight line to the data between -20 mV and -100 mV: the average of 5 consecutive ramps was calculation in each solution, except when the currents were measured immediately after patch excision when only 2 ramps were averaged to avoid errors due to channel rundown. Currents were corrected for leak (< 1% of the total current) by subtraction of the mean current recorded in Kir6.2ΔC36-injected oocytes at maximally effective concentration of ATP.

Data analysis: All data is given as mean ± S.E.M. The symbols in the figures indicate the mean and the vertical bars one S.E.M. (where this is larger than the symbol). ATP dose-response relationships were fit to the Hill equation: $G/G_c = 1 / (1 + ([ATP] / K_i)^h))$, where [ATP] is the ATP concentration, K_i is the ATP concentration at which inhibition is half maximal, and is the slope factor (Hill coefficient). Statistical significance was tested using Student's t-test.

CLAIMS:

1. A nucleic acid molecule which has a nucleotide sequence encoding a protein which is a variant form of the protein subunit of a K-ATP channel and wherein said variant protein functions as a K-ATP channel in the absence of association with a sulphonylurea receptor (SUR) protein.

2. A nucleic acid molecule according to claim 1 wherein the protein subunit has part or all of an amino acid sequence as shown in Fig 1 or Fig 2.

3. A nucleic acid molecule according to claim 1 or claim 2 wherein the variant protein is without C-terminal amino acid residues of a native protein subunit.

4. A nucleic acid molecule according to any one of claims 1 to 3 wherein the variant protein is without 1 to 36 C-terminal amino acid residues of a native protein subunit.

5. A nucleic acid molecule according to any one of claims 1 to 4 wherein the variant protein is without 1 to 26 C-terminal amino acid residues of a native protein subunit.

6. A nucleic acid molecule according to any one of claims 1 to 5 wherein the nucleotide sequence is complementary to, or as showing in Fig 4(a) or Fig 4(b) or a mutant, variant, derivative, allele or fragment of a sequence as shown in Fig 4 (a) or Fig 4(b) or of a sequence complementary to a sequence shown in Fig 4(a) or Fig 4(b).

7. A replicable cloning or expression vector which comprises a nucleic acid molecule according to any one of claims 1 to 6.

8. A host cell which comprises a replicable cloning or expression vector according to claim 7.

9. A Xenopus oocyte which expresses a nucleic molecule according to any one of claims 1 to 6 or an expression vector according to claim 7.

10. A labelled hybridization probe which comprises a nucleotide sequence which comprises part or all of a nucleotide sequence as defined in any one of claims 1 to 6.

11. An oligonucleotide primer or an oligonucleotide primer pair for amplifying a nucleic acid molecule according to any one of claims 1 to 6.

12. A polypeptide which is a variant form of the

protein subunit of a K-ATP channel and wherein said polypeptide is able to function as a K-ATP channel in the absence of association with a sulphonylurea receptor (SUR) protein.

13. A polypeptide according to claim 12 wherein the protein subunit has part or all of an amino acid sequence as shown in Fig 1 or Fig 2.

14. A polypeptide according to claim 12 or claim 13 which is without C-terminal amino acid residues of a native protein subunit.

15. A polypeptide according to any one of claims 12 to 14 which is without 1 to 36 C-terminal amino acid residues of a native protein subunit.

16. A polypeptide according to any one of claims 12 to 15 which is without 1 to 26 C-terminal amino acid residues of a native protein subunit.

17. A polypeptide according to any one of claims 12 to 16 which has a amino acid sequence as shown in Fig 4(a) or Fig 4(b) or which is an amino acid variant, allele, derivative, mutant or fragment of a polypeptide which has an amino acid sequence as shown in Fig 4(a) or Fig 4(b).

18. A binding member for a polypeptide according to any one of claims 12 to 17 which comprises an antibody binding domain specific for a said polypeptide.

19. Use of a nucleic acid molecule according to any one of claims 1 to 6 or a polypeptide according to any one of claims 12 to 17 in a method for screening for a drug.

20. Use of a nucleic acid molecule according to any one of claims 1 to 6 or a polypeptide according to any one of claims 12 to 17 in a method for screening for a drug for treatment of a disease condition associated with abnormal coupling of cellular metabolism to K^+ fluxes and/or electrical activity.

21. A drug screening method which employs a nucleic acid molecule according to any one of claims 1 to 6 or a polypeptide according to any one of claims 12 to 17.

22. A pharmaceutical preparation which comprises as an effective ingredient a drug obtainable by a method according to claim 21.

23. Use of a nucleic acid molecule according to any one of claims 1 to 6 or a polypeptide according to any one of claims 12 to 17 in the preparation of a medicament for the treatment of a disease condition selected from diabetes mellitus including non-insulin-dependent diabetes; maturity-onset-diabetes of the young and

insulin-dependent diabetes; diseases which result in increased insulin secretion such as persistent hyperinsulinaemia of infancy; hypertension and hypotension; cerebral ischaemia; stroke; cerebral vasospasm; peripheral arterial disease; vascular smooth muscle disease; angina pectoris; long QT syndrome; vasospastic angina; cardiac dysrhythmia; ischaemia induced cardiac arrhythmias; Parkinson's Disease; diseases associated with abnormal cerebellar function (eg ataxia) asthma; diseases of intestinal smooth muscle, including irritable bowel syndrome, urinary incontinence; male alopecia; certain skeletal muscle myopathies, such as those associated with membrane depolarisation, hyperpolarisation or hypopolarisation; appetite (both reduced and excessive); abnormalities and diseases of uterine smooth muscle, including premature labour; diseases involving abnormal nerve conduction; inappropriate secretion of renin and insensitivity to certain classes of drugs (eg sulphonylureas, K-channel openers etc) caused by inappropriate coupling between Kir6.2 and the drug-binding subunits of the channel complex.

24. A method for measuring submembrane ATP levels which comprises use of a nucleic acid molecule according to any one of claims 1 to 6 or of a polypeptide according to any one of claims 12 to 17.

25. Use of a nucleic acid molecule according to any one of claims 1 to 6 or of a polypeptide according to any one of claims 12 to 17 to measure submembrane ATP levels.

Fig. 1. Mouse ATP-sensitive Potassium Channel Gene (Kir6.2)

```
                                                                    ← nucleotides
GCCAAGCCAGTGTAGTGCCTCCCCCCCATGGGGGAAACCCCTTCCCGGGGCCAACGGAGCC

ATGCTGTCCCGAAAGGGCATTATCCCTGAGGAATATGTGCTGACCCGGCTGGCAGAGGAC
  M  L  S  R  K  G  I  I  P  E  E  Y  V  L  T  R  L  A  E  D   ← amino acid

CCTGCAGAGCCCAGGTACCGTACTCGAGAGAGGAGGGCCCGCTTCGTGTCCAAGAAAGGC
  P  A  E  P  R  Y  R  T  R  E  R  R  A  R  F  V  S  K  K  G

AACTGCAACGTCGCCCACAAGAACATTCGAGAGCAGGGCCGCTTCCTGCAGGATGTGTTC
  N  C  N  V  A  H  K  N  I  R  E  Q  G  R  F  L  Q  D  V  F

ACCACGCTGGTGGACCTCAAATGGCCACACACTCTGCTCATTTTCACCATGTCCTTCCTG
  T  T  L  V  D  L  K  W  P  H  T  L  L  I  F  T  M  S  F  L

TGCAGCTGGCTGCTCTTTGCCATGGTCTGGTGGCTCATCGCCTTCGCCCACGGTGACCTG
  C  S  W  L  L  F  A  M  V  W  W  L  I  A  F  A  H  G  D  L

GCCCCCGGAGAGGGCACCAATGTGCCCTGCGTCACAAGCATCCACTCCTTTTCATCTGCC
  A  P  G  E  G  T  N  V  P  C  V  T  S  I  H  S  F  S  S  A

TTCCTTTTCTCCATCGAGGTCCAGGTGACCATTGGTTTCGGCGGGCGCATGGTGACAGAG
  F  L  F  S  I  E  V  Q  V  T  I  G  F  G  G  R  M  V  T  E

GAATGTCCCCTGGCCATCCTCATTCTCATTGTGCAGAATATCGTCGGGCTGATGATCAAC
  E  C  P  L  A  I  L  I  I  V  Q  N  I  V  G  L  M  I  N

GCCATCATGCTGGGCTGCATCTTCATGAAAACGGCCCAGGCCCATCGGCGGGCAGAAACC
  A  I  M  L  G  C  I  F  M  K  T  A  Q  A  H  R  R  A  E  T

CTCATCTTCAGCAAGCATGCTGTGATCACCCTGCGCCATGGCCGCCTGTGCTTCATGCTG
  L  I  F  S  K  H  A  V  I  T  L  R  H  G  R  L  C  F  M  L
```

Fig.1 (Cont).

```
CGCGTAGGGGACCTCCGAAAGAGCATGATCATTAGCGCCACCATCCACATGCAGGTGGTG
  R  V  G  D  L  R  K  S  M  I  I  S  A  T  I  H  M  Q  V  V

CGCAAGACCACCAGCCCCGAGGGCGAAGTTGTGCCTCTCCACCAGGTAGACATCCCCATG
  R  K  T  T  S  P  E  G  E  V  V  P  L  H  Q  V  D  I  P  M

GAGAATGGCGTGGGTGGTAACGGCATCTTCCTGGTGGCCCCACTCATCATCTACCACGTC
  E  N  G  V  G  G  N  G  I  F  L  V  A  P  L  I  I  Y  H  V

ATCGACTCCAACAGCCCGCTCTACGACCTGGCTCCTAGTGACCTGCACCACCACCAGGAC
  I  D  S  N  S  P  L  Y  D  L  A  P  S  D  L  H  H  H  Q  D

CTGGAGATCATTGTCATCTTGGAAGGCGTGGTAGAAACCACGGGCATCACCACCCAGGCC
  L  E  I  I  V  I  L  E  G  V  V  E  T  T  G  I  T  T  Q  A

CGCACCTCCTACCTAGCTGACGAGATTCTATGGGGGCAGCGCTTTGTCCCCATTGTGGCC
  R  T  S  Y  L  A  D  E  I  L  W  G  Q  R  F  V  P  I  V  A

GAGGAGGACGGCCGCTATTCTGTGGACTACTCCAAATTTGGTAACACCATTAAAGTGCCC
  E  E  D  G  R  Y  S  V  D  Y  S  K  F  G  N  T  I  K  V  P

ACACCACTCTGCACAGCCCGCCAGCTTGATGAGGACCGCAGTCTGCTGGATGCCCTGACC
  T  P  L  C  T  A  R  Q  L  D  E  D  R  S  L  L  D  A  L  T

CTCGCCTCGTCGCGGGGGCCCCTGCGCAAGCGCAGTGTGGCTGTGGCGAAGGCCAAGCCC
  L  A  S  S  R  G  P  L  R  K  R  S  V  A  V  A  K  A  K  P

AAGTTTAGCATCTCTCCAGATTCCTTGTCCTGAGTTGCAGTTCCTCAGGCCCCCACTCAC
  K  F  S  I  S  P  D  S  L  S  *

TTGTGTGGGCACGTGGAAAGTGAAGTATGGTATGTAGAGTGGTGGGGTGCGAGCCTCTTG

GCCAGACGAGGG
```

Fig.2.

Human Kir6.2 gene

```
                                                GGTGCCTCCGATGGGGGAAGCCCCTCCCTGGGGGTCACCGGAGCC

ATGCTGTCCCGCAAGGGCATCATCCCCGAGGAATACGTGCTGACACGCCTGGCAGAGGAC
 M  L  S  R  K  G  I  I  P  E  E  Y  V  L  T  R  L  A  E  D

CCTGCCGAGCCCAGGTACCGTGCCCGCCAGCGGAGGGCCCGCTTTGTGTCCAAGAAAGGC
 P  A  E  P  R  Y  R [A] R [Q] R  R  A  R  F  V  S  K  K  G

AACTGCAACGTGGCCCACAAGAACATCCGGGAGCAGGGCCGCTTCCTGCAGGACGTGTTC
 N  C  N  V  A  H  K  N  I  R  E  Q  G  R  F  L  Q  D  V  F

ACCACGCTGGTGGACCTCAAGTGGCCACACACATTGCTCATCTTCACCATGTCCTTCCTG
 T  T  L  V  D  L  K  W  P  H  T  L  L  I  F  T  M  S  F  L

TGCAGCTGGCTGCTCTTCGCCATGGCCTGGTGGCTCATCGCCTTCGCCCACGGTGACCTG
 C  S  W  L  L  F  A  M [A] W  W  L  I  A  F  A  H  G  D  L

GCCCCCAGCGAGGGCACTGCTGAGCCCTGTGTCACCAGCATCCACTCCTTCTCGTCTGCC
 A  P [S] E  G  T [A][E] P  C  V  T  S  I  H  S  F  S  S  A

TTCCTTTTCTCCATTGAGGTCCAAGTGACTATTGGCTTTGGGGGGCGCATGGTGACTGAG
 F  L  F  S  I  E  V  Q  V  T  I  G  F  G  G  R  M  V  T  E

GAGTGCCCACTGGCCATCCTGATCCTCATCGTGCAGAACATCGTGGGGCTCATGATCAAC
 E  C  P  L  A  I  L  I  L  I  V  Q  N  I  V  G  L  M  I  N

GCCATCATGCTTGGCTGCATCTTCATGAAGACTGCCCAAGCCCACCGCAGGGCTGAGACC
 A  I  M  L  G  C  I  F  M  K  T  A  Q  A  H  R  R  A  E  T

CTCATCTTCAGCAAGCATGCGGTGATCGCTCTGCGCCACGGCCGCCTCTGCTTCATGCTA
 L  I  F  S  K  H  A  V  I [A] L  R  H  G  R  L  C  F  M  L

CGTGTGGGTGACCTCCGCAAGAGCATGATCATCAGCGCCACCATCCACATGCAGGTGGTA
 R  V  G  D  L  R  K  S  M  I  I  S  A  T  I  H  M  Q  V  V

CGCAAGACCACCAGCCCCGAGGGCGAGGTGGTGCCCCTCCACCAGGTGGACATCCCCATG
 R  K  T  T  S  P  E  G  E  V  V  P  L  H  Q  V  D  I  P  M

GAGAACGGCGTGGGTGGCAACAGCATCTTCCTGGTGGCCCCGCTGATCATCTACCATGTC
 E  N  G  V  G  G  N [S] I  F  L  V  A  P  L  I  I  Y  H  V

ATTGATGCCAACAGCCCACTCTACGACCTGGCACCCAGCGACCTGCACCACCACCAGGAC
 I  D [A] N  S  P  L  Y  D  L  A  P  S  D  L  H  H  H  Q  D

CTCGAGATCATCGTCATCCTGGAAGGCGTGGTGGAAACCACGGGCATCACCACCCAGGCC
 L  E  I  I  V  I  L  E  G  V  V  E  T  T  G  I  T  T  Q  A

CGCACCTCCTACCTGGCCGATGAGATCCTGTGGGGCCAGCGCTTTGTGCCCATTGTAGCT
 R  T  S  Y  L  A  D  E  I  L  W  G  Q  R  F  V  P  I  V  A
```

Fig.2 (Cont).

```
GAGGAGGACGGACGTTACTCTGTGGACTACTCCAAGTTTGGCAACACCATCAAAGTGCCC
 E  E  D  G  R  Y  S  V  D  Y  S  K  F  G  N  T  I  K  V  P

ACACCACTCTGCACGGCCCGCCAGCTTGATGAGGACCACAGCCTACTGGAAGCTCTGACC
 T  P  L  C  T  A  R  Q  L  D  E  D [H] S  L  L [E] A  L  T

CTCGCCTCAGCCCGCGGGCCCCTGCGCAAGCGCAGCGTGCCCATGGCCAAGGCCAAGCCC
 L  A  S [A] R  G  P  L  R  K  R  S  V [P][M] A  K  A  K  P

AAGTTCAGCATCTCTCCAGATTCCCTGTCCTGAGCCATGGTCTCTCGGGCCCCCCACACG
 K  F  S  I  S  P  D  S  L  S  *

CGTGTGTACACACGGACCATGTGGTATGTAGCC
```

[ ] Marks those amino acids which differ from the amino acids present at equivalent residues in the mouse sequence as shown in Fig.1

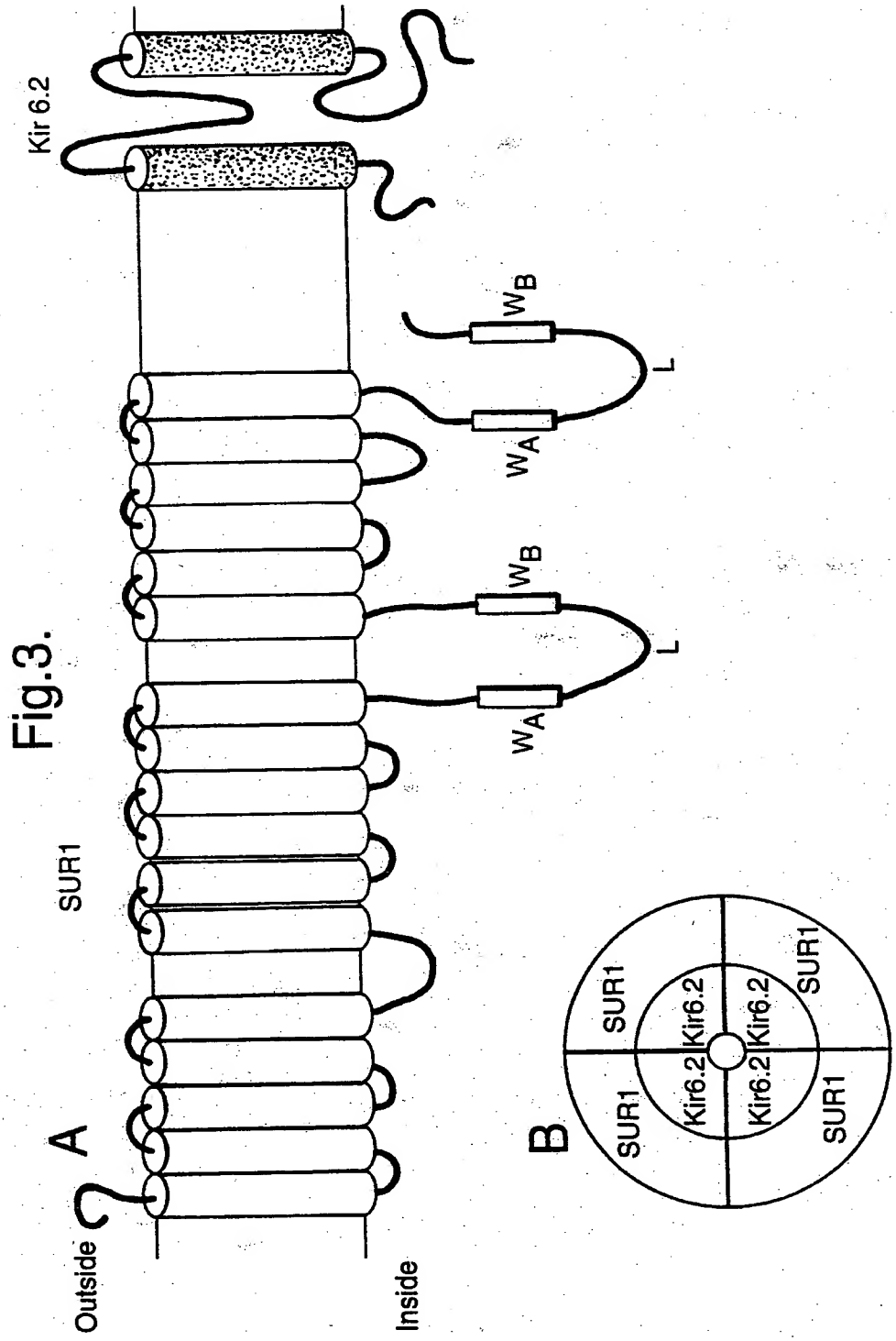

Kir 6.2
SUR1
A
Outside
Inside
W_B
W_A
L
W_B
W_A
L
B
SUR1
Kir6.2
Kir6.2
SUR1
Kir6.2
Kir6.2
SUR1
SUR1

Fig.3.

Fig.4(a).

Human Kir6.2 gene Variant (Kir6.2ΔC26)

```
                                                GGTGCCTCCGATGGGGGAAGCCCCTCCCTGGGGGTCACCGGAGCC

ATGCTGTCCCGCAAGGGCATCATCCCCGAGGAATACGTGCTGACACGCCTGGCAGAGGAC
 M  L  S  R  K  G  I  I  P  E  E  Y  V  L  T  R  L  A  E  D

CCTGCCGAGCCCAGGTACCGTGCCCGCCAGCGGAGGGCCCGCTTTGTGTCCAAGAAAGGC
 P  A  E  P  R  Y  R [A] R [Q] R  R  A  R  F  V  S  K  K  G

AACTGCAACGTGGCCCACAAGAACATCCGGGAGCAGGGCCGCTTCCTGCAGGACGTGTTC
 N  C  N  V  A  H  K  N  I  R  E  Q  G  R  F  L  Q  D  V  F

ACCACGCTGGTGGACCTCAAGTGGCCACACACATTGCTCATCTTCACCATGTCCTTCCTG
 T  T  L  V  D  L  K  W  P  H  T  L  L  I  F  T  M  S  F  L

TGCAGCTGGCTGCTCTTCGCCATGGCCTGGTGGCTCATCGCCTTCGCCCACGGTGACCTG
 C  S  W  L  L  F  A  M [A] W  W  L  I  A  F  A  H  G  D  L

GCCCCCAGCGAGGGCACTGCTGAGCCCTGTGTCACCAGCATCCACTCCTTCTCGTCTGCC
 A  P [S] E  G  T [A][E] P  C  V  T  S  I  H  S  F  S  S  A

TTCCTTTTCTCCATTGAGGTCCAAGTGACTATTGGCTTTGGGGGGCGCATGGTGACTGAG
 F  L  F  S  I  E  V  Q  V  T  I  G  F  G  G  R  M  V  T  E

GAGTGCCCACTGGCCATCCTGATCCTCATCGTGCAGAACATCGTGGGGCTCATGATCAAC
 E  C  P  L  A  I  L  I  L  I  V  Q  N  I  V  G  L  M  I  N

GCCATCATGCTTGGCTGCATCTTCATGAAGACTGCCCAAGCCCACCGCAGGGCTGAGACC
 A  I  M  L  G  C  I  F  M  K  T  A  Q  A  H  R  R  A  E  T

CTCATCTTCAGCAAGCATGCGGTGATCGCTCTGCGCCACGGCCGCCTCTGCTTCATGCTA
 L  I  F  S  K  H  A  V  I [A] L  R  H  G  R  L  C  F  M  L

CGTGTGGGTGACCTCCGCAAGAGCATGATCATCAGCGCCACCATCCACATGCAGGTGGTA
 R  V  G  D  L  R  K  S  M  I  I  S  A  T  I  H  M  Q  V  V

CGCAAGACCACCAGCCCCGAGGGCGAGGTGGTGCCCCTCCACCAGGTGGACATCCCCATG
 R  K  T  T  S  P  E  G  E  V  V  P  L  H  Q  V  D  I  P  M

GAGAACGGCGTGGGTGGCAACAGCATCTTCCTGGTGGCCCCGCTGATCATCTACCATGTC
 E  N  G  V  G  G  N [S] I  F  L  V  A  P  L  I  I  Y  H  V

ATTGATGCCAACAGCCCACTCTACGACCTGGCACCCAGCGACCTGCACCACCACCAGGAC
 I  D [A] N  S  P  L  Y  D  L  A  P  S  D  L  H  H  H  Q  D

CTCGAGATCATCGTCATCCTGGAAGGCGTGGTGGAAACCACGGGCATCACCACCCAGGCC
 L  E  I  I  V  I  L  E  G  V  V  E  T  T  G  I  T  T  Q  A

CGCACCTCCTACCTGGCCGATGAGATCCTGTGGGGCCAGCGCTTTGTGCCCATTGTAGCT
 R  T  S  Y  L  A  D  E  I  L  W  G  Q  R  F  V  P  I  V  A
```

Fig.4(a) (Cont).

GAGGAGGACGGACGTTACTCTGTGGACTACTCCAAGTTTGGCAACACCATCAAAGTGCCC
E E D G R Y S V D Y S K F G N T I K V P

ACACCACTCTGCACGGCCCGCCAGCTTGATGAGGACCACAGCCTACTGGAAGCTCTGACC
T P L C T A R Q L D E D [H] S L L [E] A L T

CTCGCCTCAGCC
L A S [A]

☐ Marks those amino acids which differ from the amino acids present at equivalent residues in the mouse sequence as shown in Fig.1

Human Kir6.2 gene Variant (Kir6.2ΔC36) Fig.4(b).

```
                                                GGTGCCTCCGATGGGGGAAGCCCCTCCCTGGGGGTCACCGGAGCC

ATGCTGTCCCGCAAGGGCATCATCCCCGAGGAATACGTGCTGACACGCCTGGCAGAGGAC
 M  L  S  R  K  G  I  I  P  E  E  Y  V  L  T  R  L  A  E  D

CCTGCCGAGCCCAGGTACCGTGCCCGCCAGCGGAGGGCCCGCTTTGTGTCCAAGAAAGGC
 P  A  E  P  R  Y  R [A] R [Q] R  R  A  R  F  V  S  K  K  G

AACTGCAACGTGGCCCACAAGAACATCCGGGAGCAGGGCCGCTTCCTGCAGGACGTGTTC
 N  C  N  V  A  H  K  N  I  R  E  Q  G  R  F  L  Q  D  V  F

ACCACGCTGGTGGACCTCAAGTGGCCACACACATTGCTCATCTTCACCATGTCCTTCCTG
 T  T  L  V  D  L  K  W  P  H  T  L  L  I  F  T  M  S  F  L

TGCAGCTGGCTGCTCTTCGCCATGGCCTGGTGGCTCATCGCCTTCGCCCACGGTGACCTG
 C  S  W  L  L  F  A  M [A] W  W  L  I  A  F  A  H  G  D  L

GCCCCCAGCGAGGGCACTGCTGAGCCCTGTGTCACCAGCATCCACTCCTTCTCGTCTGCC
 A  P [S] E  G  T [A][E] P  C  V  T  S  I  H  S  F  S  S  A

TTCCTTTTCTCCATTGAGGTCCAAGTGACTATTGGCTTTGGGGGGCGCATGGTGACTGAG
 F  L  F  S  I  E  V  Q  V  T  I  G  F  G  G  R  M  V  T  E

GAGTGCCCACTGGCCATCCTGATCCTCATCGTGCAGAACATCGTGGGGCTCATGATCAAC
 E  C  P  L  A  I  L  I  L  I  V  Q  N  I  V  G  L  M  I  N

GCCATCATGCTTGGCTGCATCTTCATGAAGACTGCCCAAGCCCACCGCAGGGCTGAGACC
 A  I  M  L  G  C  I  F  M  K  T  A  Q  A  H  R  R  A  E  T

CTCATCTTCAGCAAGCATGCGGTGATCGCTCTGCGCCACGGCCGCCTCTGCTTCATGCTA
 L  I  F  S  K  H  A  V  I [A] L  R  H  G  R  L  C  F  M  L

CGTGTGGGTGACCTCCGCAAGAGCATGATCATCAGCGCCACCATCCACATGCAGGTGGTA
 R  V  G  D  L  R  K  S  M  I  I  S  A  T  I  H  M  Q  V  V

CGCAAGACCACCAGCCCCGAGGGCGAGGTGGTGCCCCTCCACCAGGTGGACATCCCCATG
 R  K  T  T  S  P  E  G  E  V  V  P  L  H  Q  V  D  I  P  M

GAGAACGGCGTGGGTGGCAACAGCATCTTCCTGGTGGCCCCGCTGATCATCTACCATGTC
 E  N  G  V  G  G  N [S] I  F  L  V  A  P  L  I  I  Y  H  V

ATTGATGCCAACAGCCCACTCTACGACCTGGCACCCAGCGACCTGCACCACCACCAGGAC
 I  D [A] N  S  P  L  Y  D  L  A  P  S  D  L  H  H  H  Q  D

CTCGAGATCATCGTCATCCTGGAAGGCGTGGTGGAAACCACGGGCATCACCACCCAGGCC
 L  E  I  I  V  I  L  E  G  V  V  E  T  T  G  I  T  T  Q  A

CGCACCTCCTACCTGGCCGATGAGATCCTGTGGGGCCAGCGCTTTGTGCCCATTGTAGCT
 R  T  S  Y  L  A  D  E  I  L  W  G  Q  R  F  V  P  I  V  A

GAGGAGGACGGACGTTACTCTGTGGACTACTCCAAGTTTGGCAACACCATCAAAGTGCCC
 E  E  D  G  R  Y  S  V  D  Y  S  K  F  G  N  T  I  K  V  P

ACACCACTCTGCACGGCCCGCCAGCTTGATGAGGACCACAGC
 T  P  L  C  T  A  R  Q  L  D  E  D [H] S
```

Fig.5.

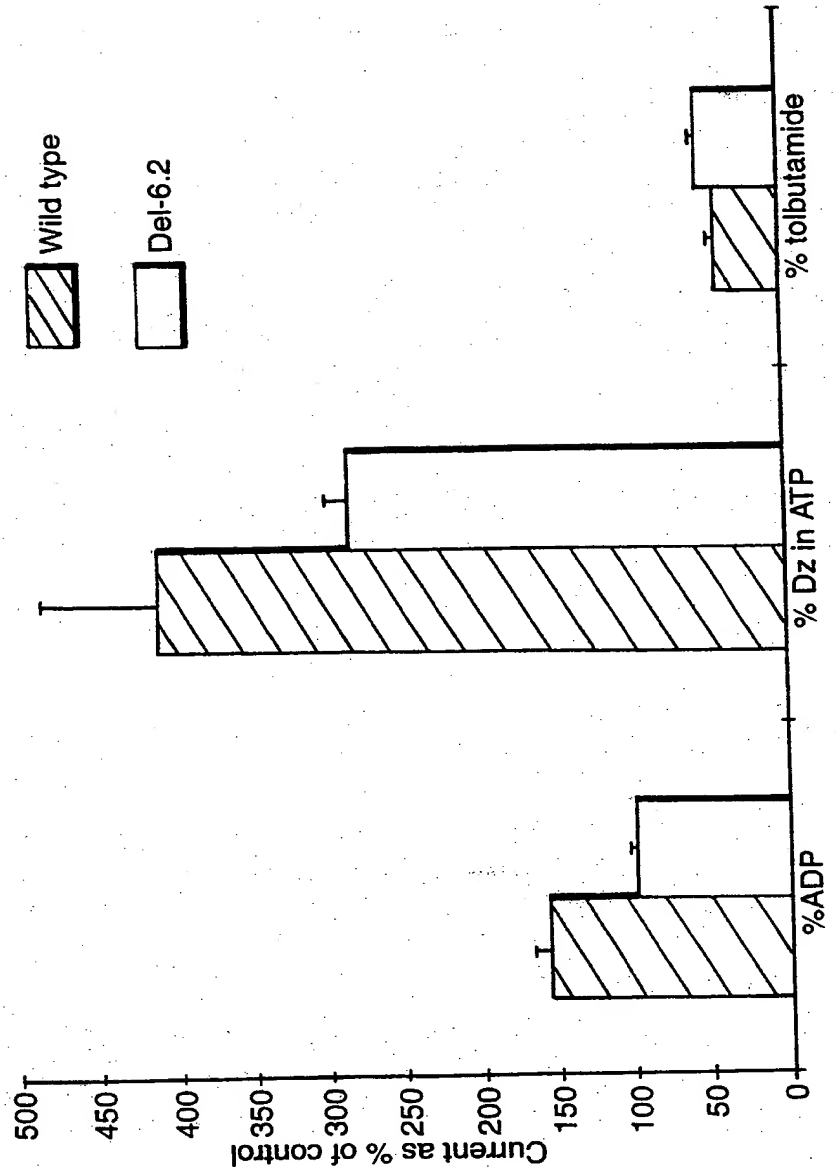

Wild type
Del-6.2
Current as % of control
500
450
400
350
300
250
200
150
100
50
0
%ADP
% Dz in ATP
% tolbutamide

Fig.6.

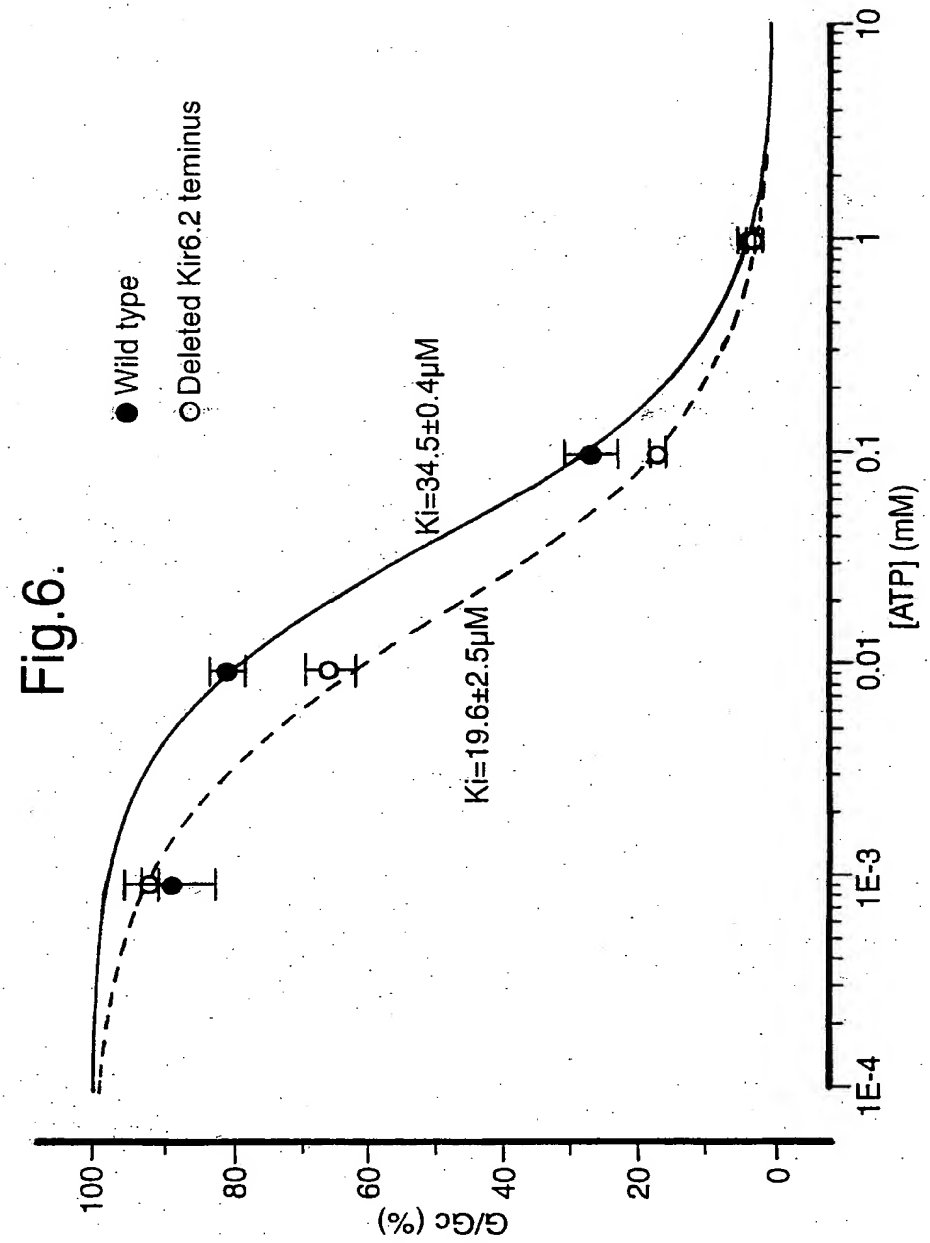

Wild type
Deleted Kir6.2 teminus
Ki=34.5±0.4μM
Ki=19.6±2.5μM
G/Gc (%)
100
80
60
40
20
0
1E-4
1E-3
0.01
0.1
1
10
[ATP] (mM)

Fig.7.

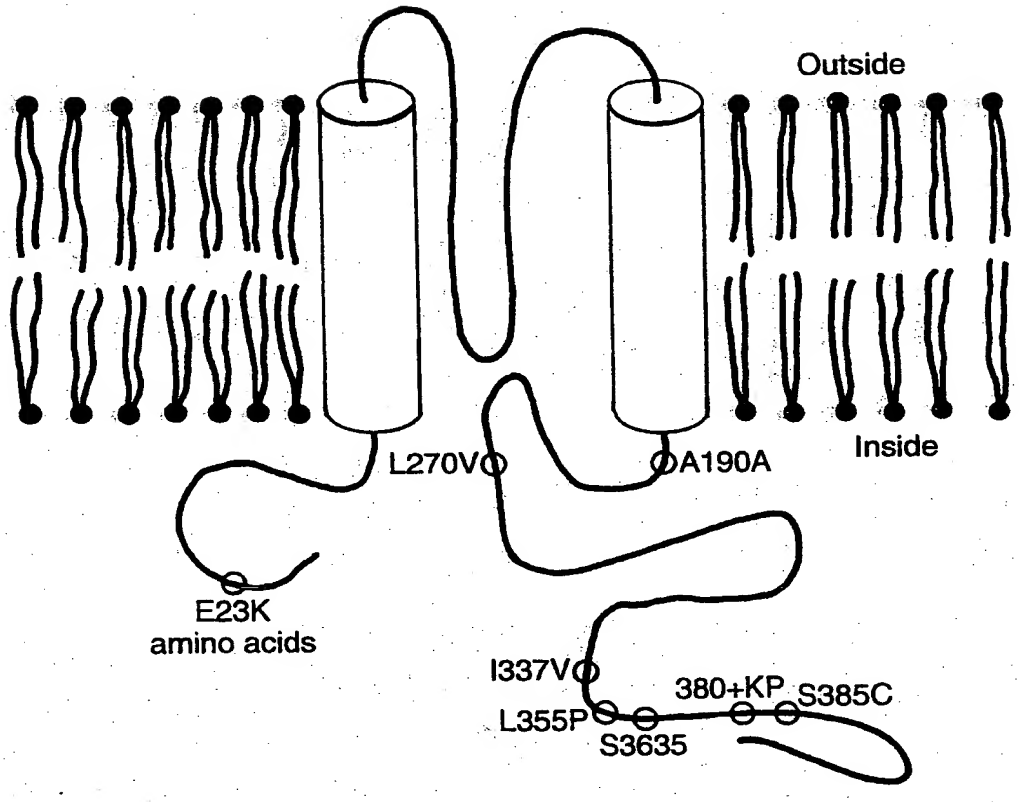

Outside
Inside
L270V
A190A
E23K
amino acids
I337V
380+KP
S385C
L355P
S3635

Fig.8.

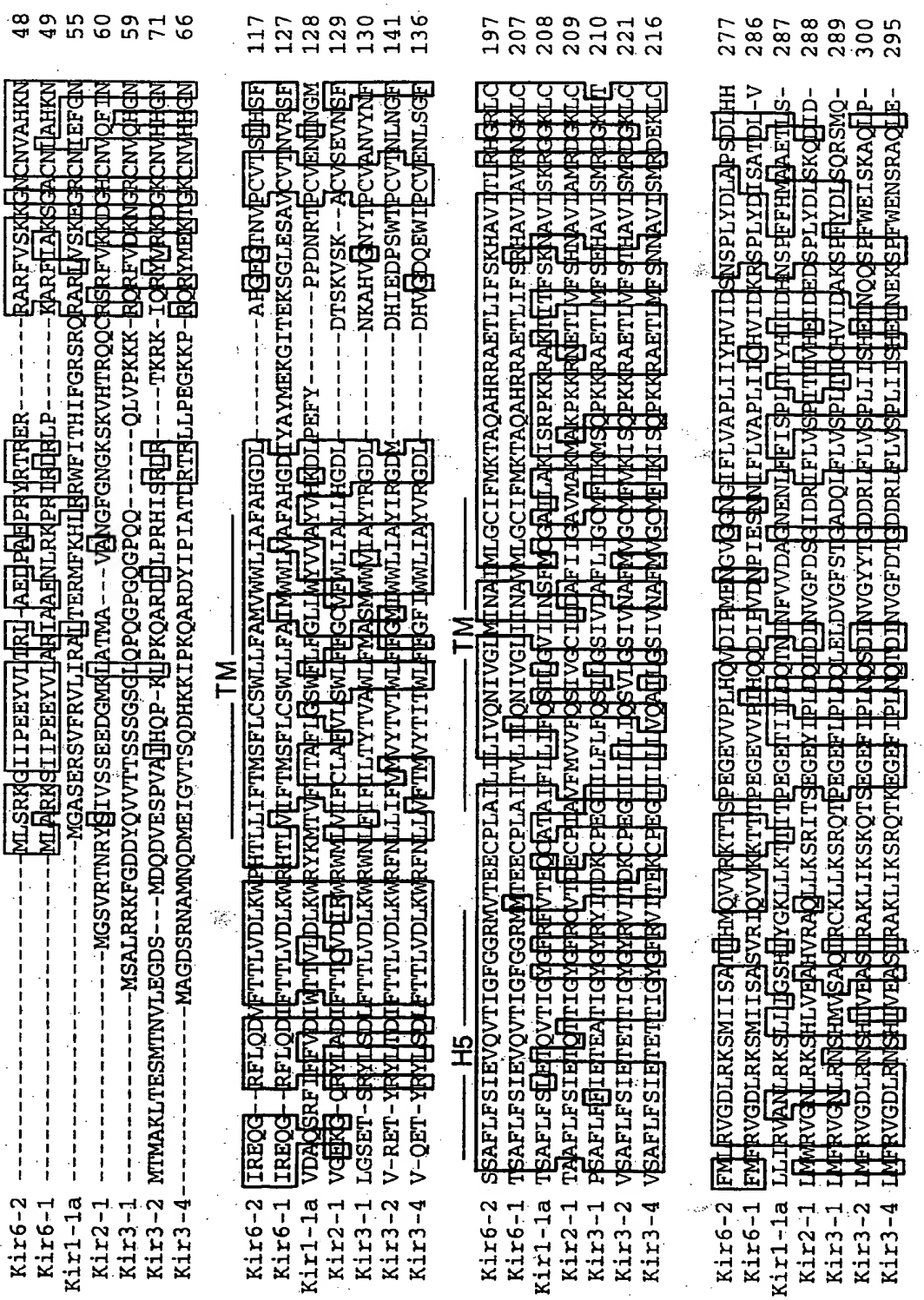


Fig.8 (Cont).

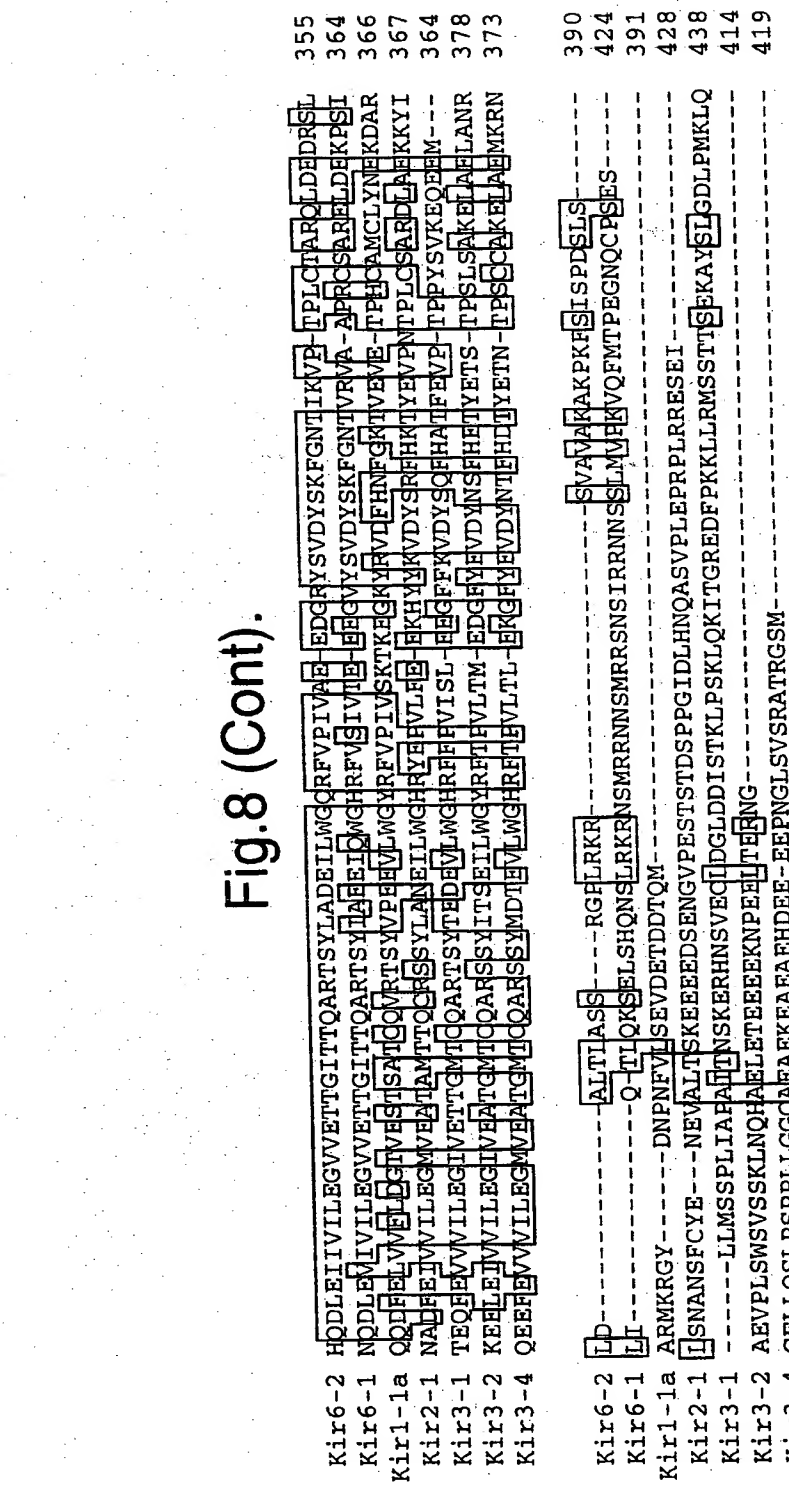


Fig.9.

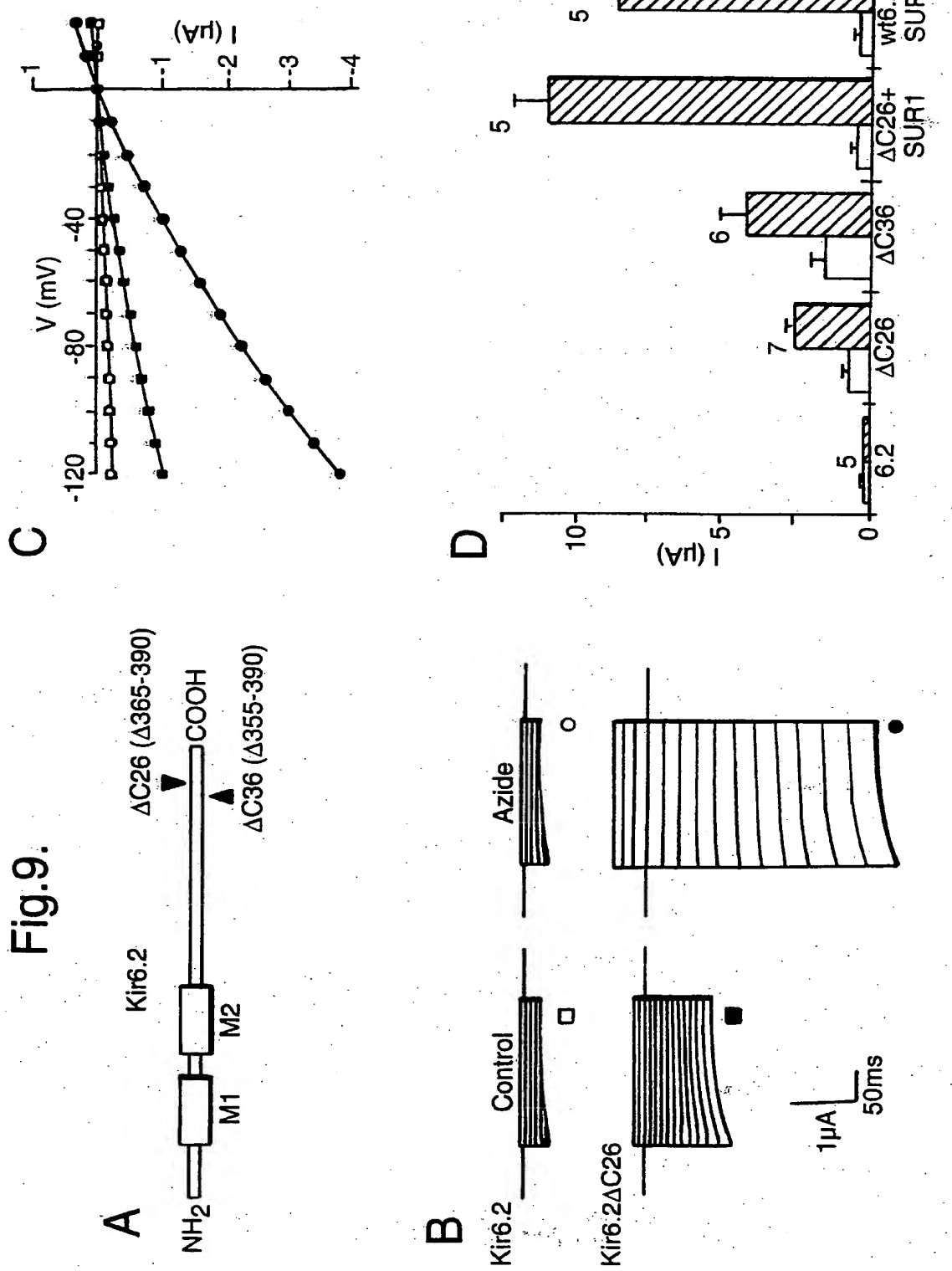
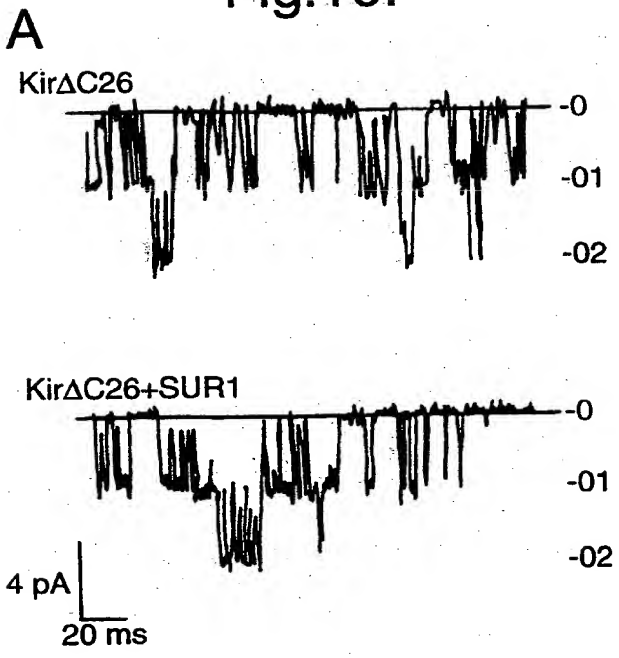

A
Kir6.2
NH2
M1
M2
COOH
ΔC26 (Δ365-390)
ΔC36 (Δ355-390)
B
Kir6.2
Control
Azide
Kir6.2ΔC26
1μA
50ms
C
V (mV)
-120
-80
-40
I (μA)
-1
-2
-3
-4
D
I (μA)
0
5
10
6.2
ΔC26
ΔC36
ΔC26+ SUR1
wt6.2+ SUR1

Fig.10.


A
KirΔC26
-0
-01
-02
KirΔC26+SUR1
-0
-01
-02
4 pA
20 ms

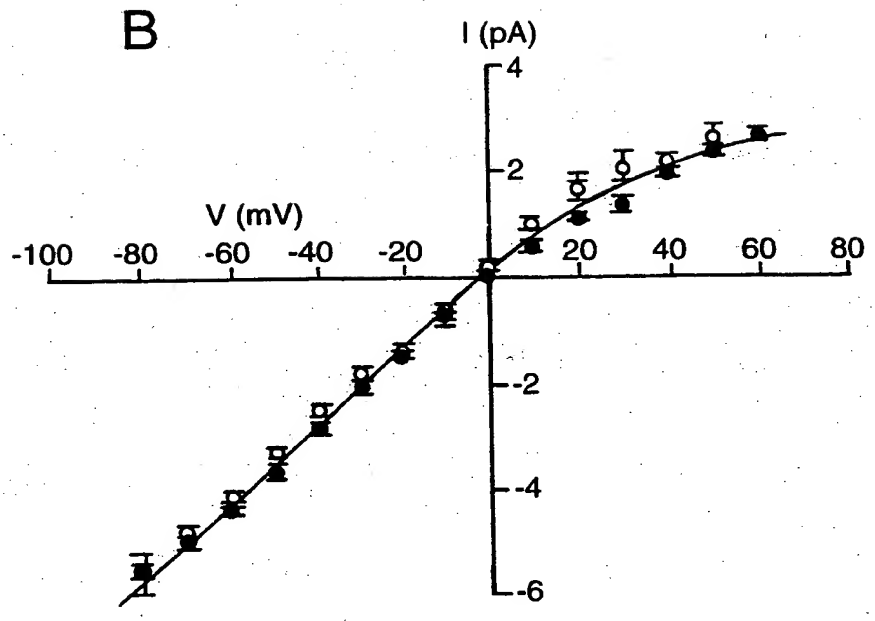

B
I (pA)
4
2
V (mV)
-100
-80
-60
-40
-20
20
40
60
80
-2
-4
-6

Fig.11.

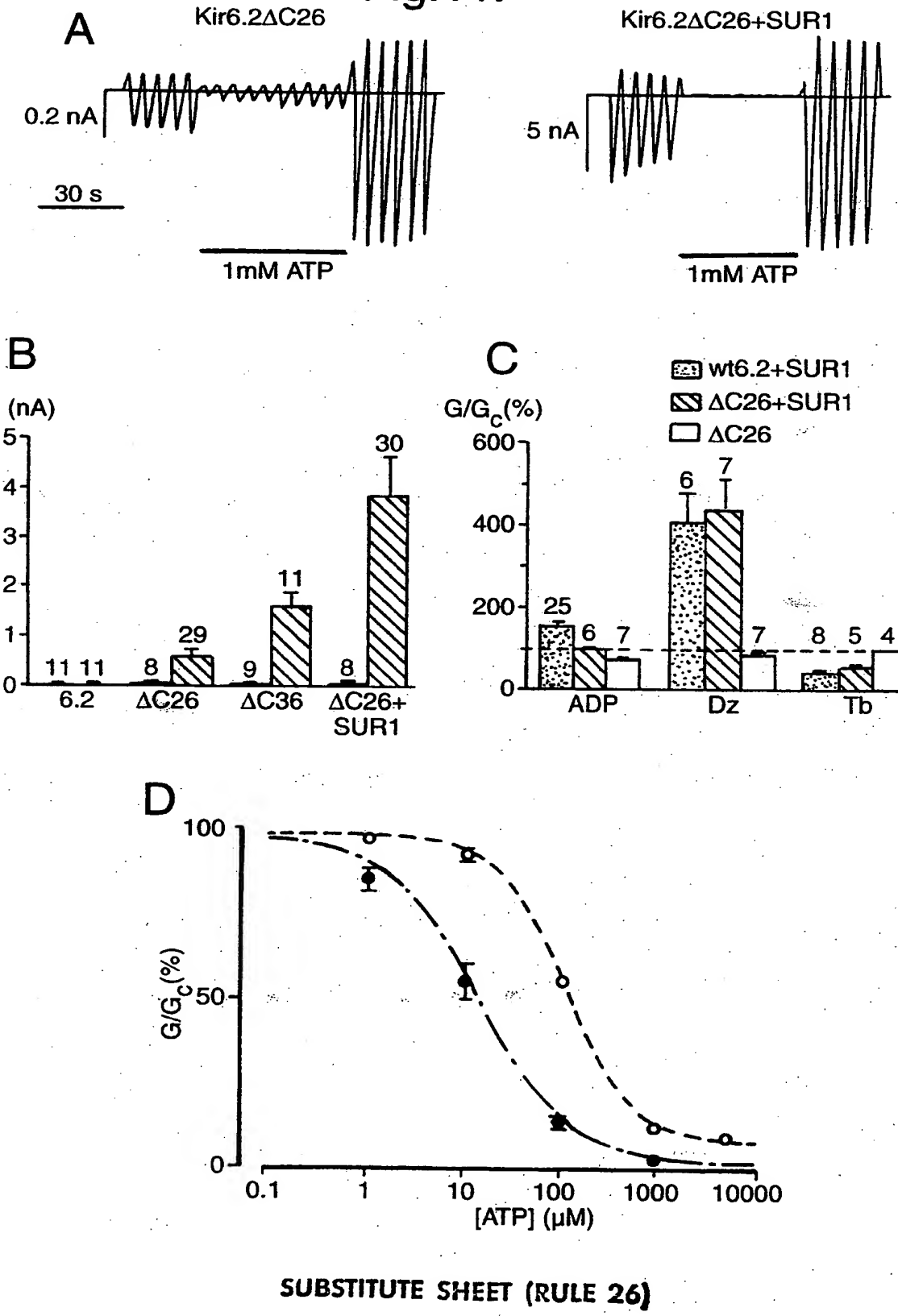

A
Kir6.2ΔC26
0.2 nA
30 s
1mM ATP
Kir6.2ΔC26+SUR1
5 nA
1mM ATP
B
I (nA)
5
4
3
2
1
0
11 11
8
29
9
11
8
30
6.2
ΔC26
ΔC36
ΔC26+ SUR1
C
G/Gc(%)
600
400
200
0
wt6.2+SUR1
ΔC26+SUR1
ΔC26
25
6
7
6
7
7
8
5
4
ADP
Dz
Tb
D
100
50
0
G/Gc(%)
0.1
1
10
100
1000
10000
[ATP] (μM)

Fig.12.

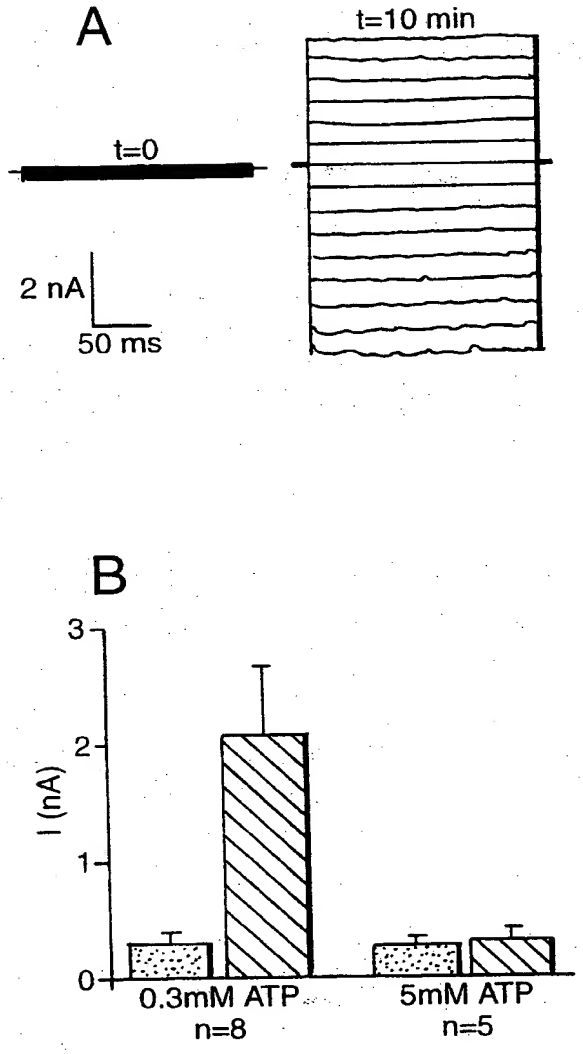


Fig.13.

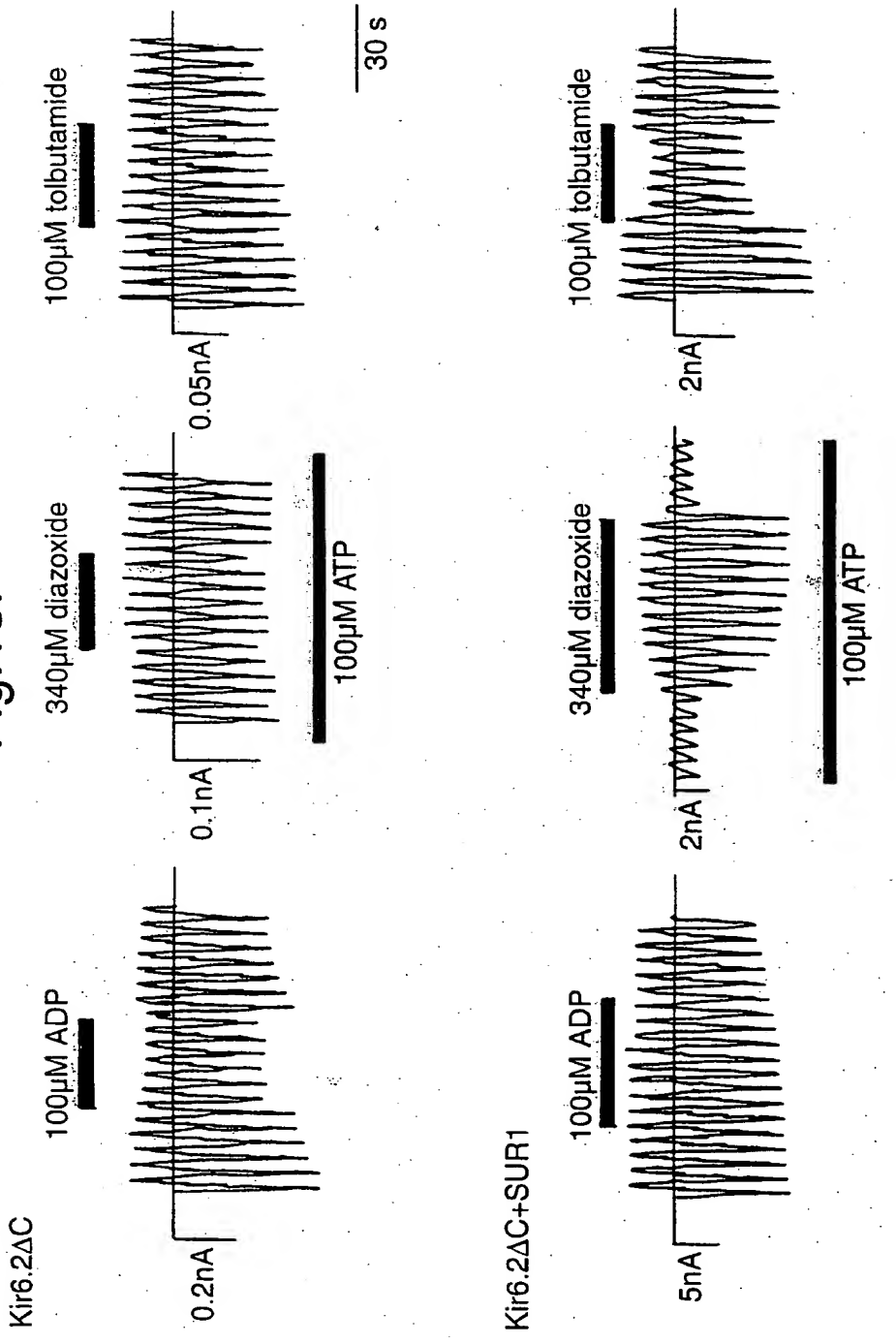

Kir6.2ΔC
100μM ADP
0.2nA
340μM diazoxide
0.1nA
100μM ATP
100μM tolbutamide
0.05nA
30 s
Kir6.2ΔC+SUR1
100μM ADP
5nA
340μM diazoxide
2nA
100μM ATP
100μM tolbutamide
2nA

Fig.14.

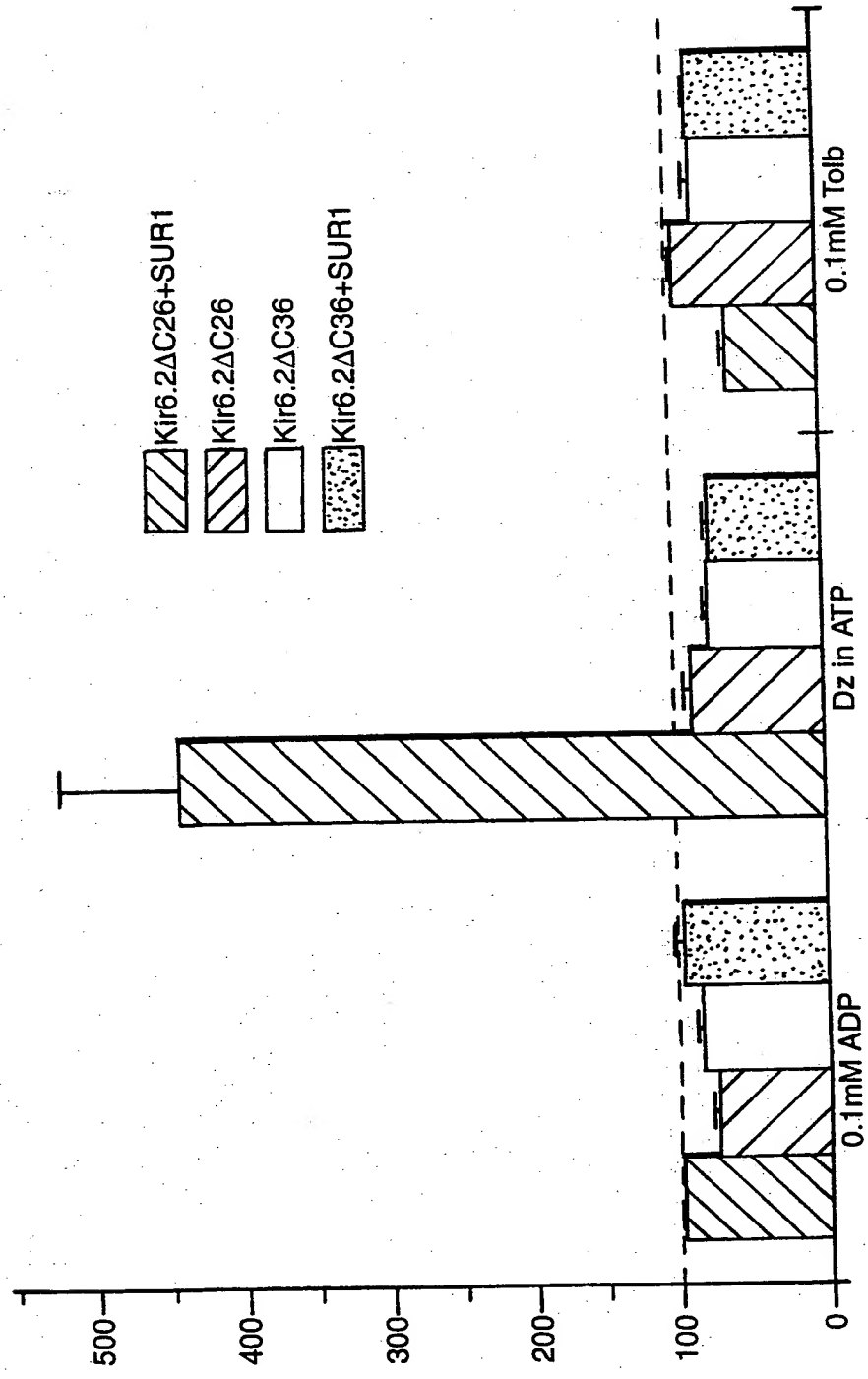
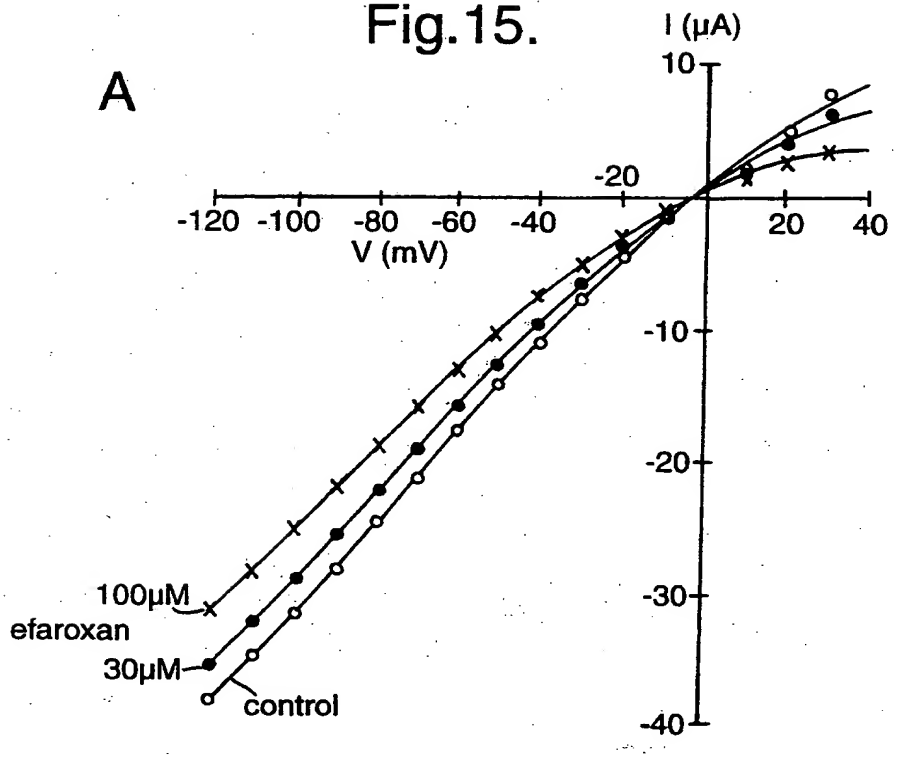

Kir6.2ΔC26+SUR1
Kir6.2ΔC26
Kir6.2ΔC36
Kir6.2ΔC36+SUR1
0.1mM Tolb
Dz in ATP
0.1mM ADP
500
400
300
200
100
0

Fig.15.


A
I (μA)
10
-20
-120 -100 -80 -60 -40
V (mV)
20 40
-10
-20
-30
-40
100μM
efaroxan
30μM
control

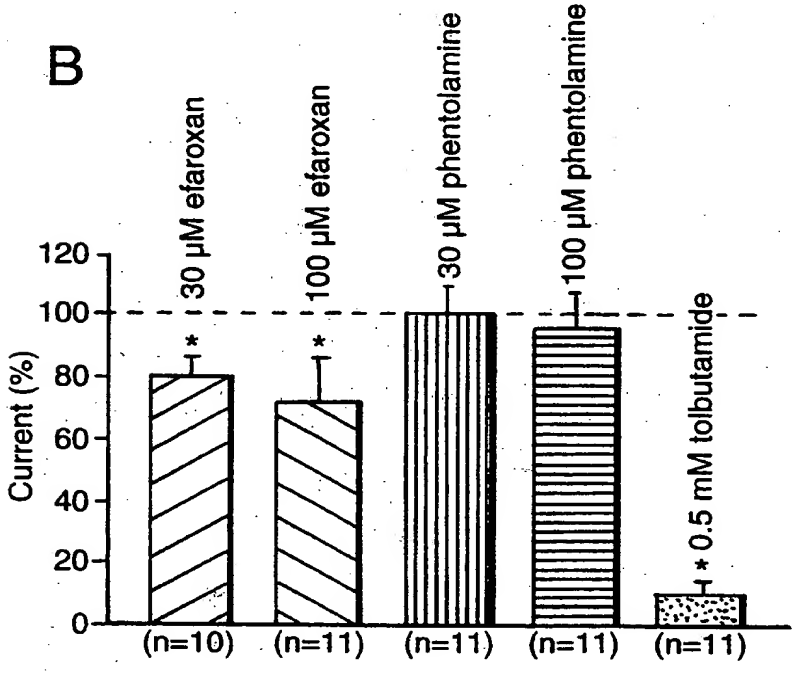

B
30 μM efaroxan
100 μM efaroxan
30 μM phentolamine
100 μM phentolamine
0.5 mM tolbutamide
Current (%)
120
100
80
60
40
20
0
(n=10)
(n=11)
(n=11)
(n=11)
(n=11)

Fig.16.

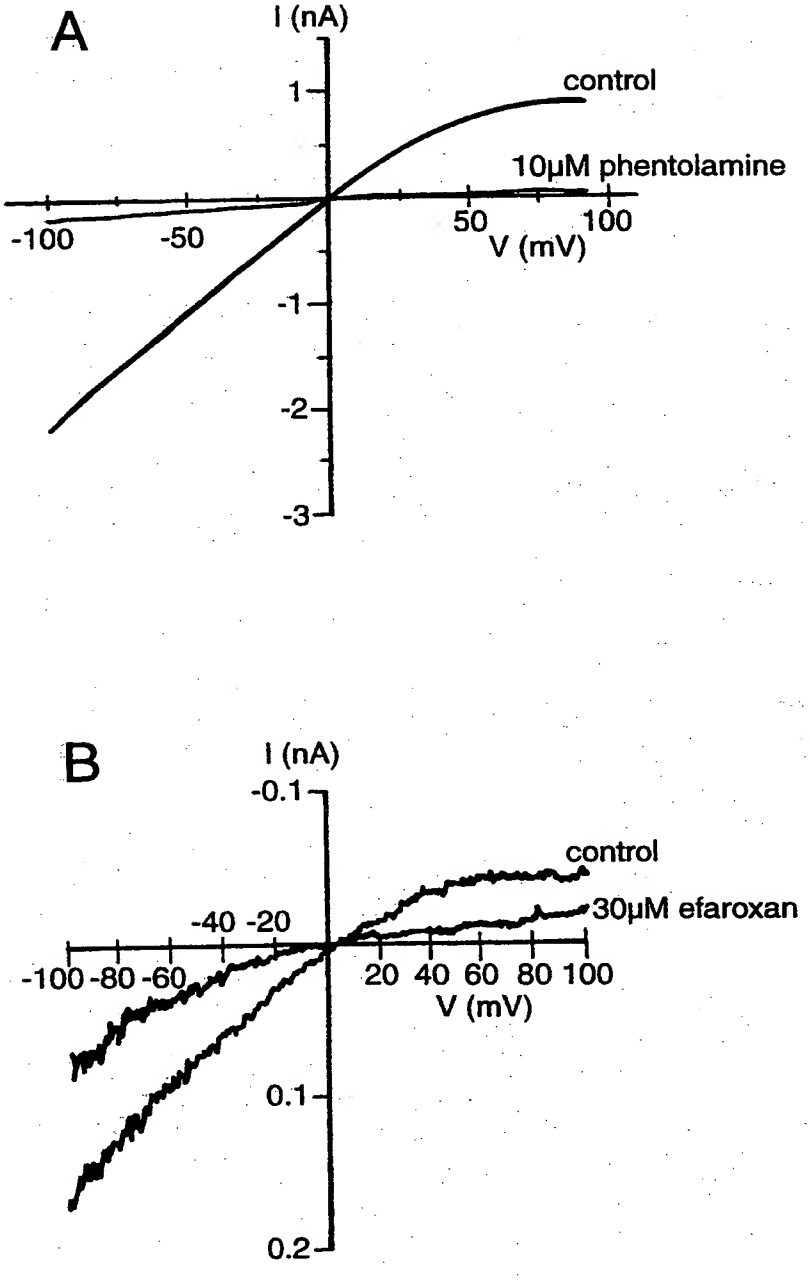

A
I (nA)
1
control
10μM phentolamine
-100
-50
50
100
V (mV)
-1
-2
-3
B
I (nA)
-0.1
control
30μM efaroxan
-40 -20
-100 -80 -60
20 40 60 80 100
V (mV)
0.1
0.2

Fig.17.

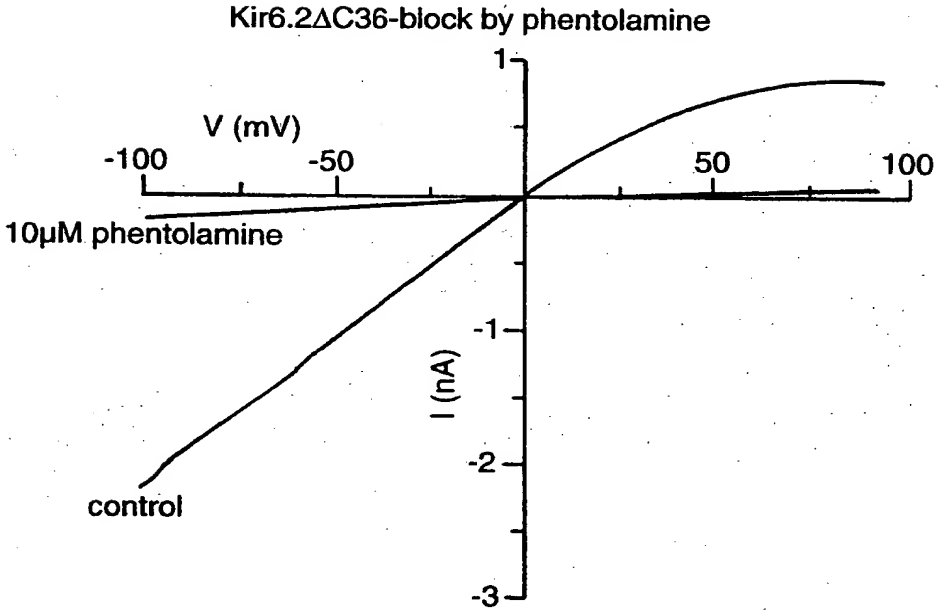
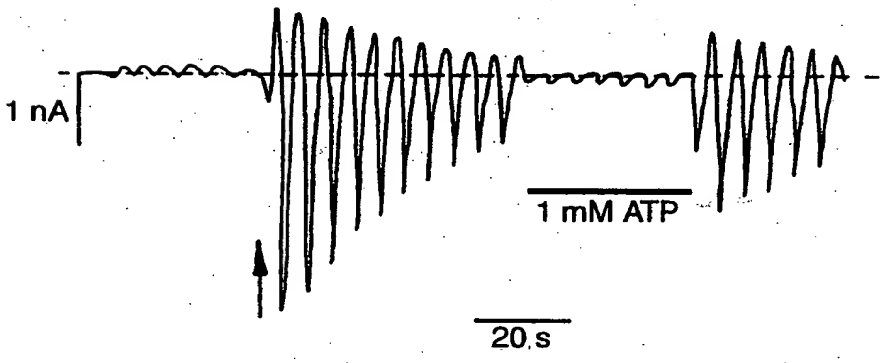

Kir6.2ΔC36-block by phentolamine
V (mV)
-100
-50
50
100
1
-1
-2
-3
I (nA)
10μM phentolamine
control

Fig.20.


Kir6.2ΔC36
1 nA
1 mM ATP
20 s

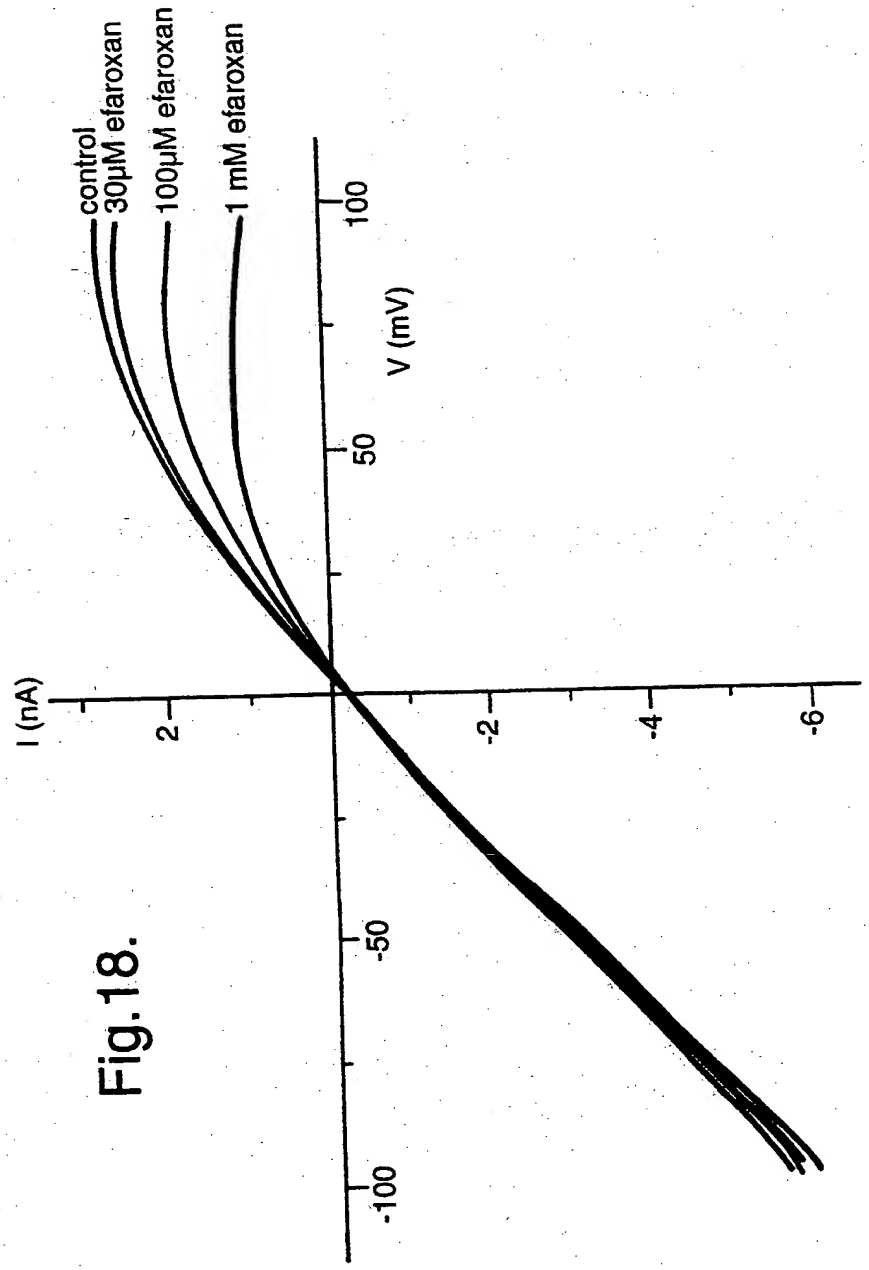

control
30µM efaroxan
100µM efaroxan
1 mM efaroxan
I (nA)
2
-2
-4
-6
V (mV)
100
50
-50
-100

Fig.18.

Fig.19.

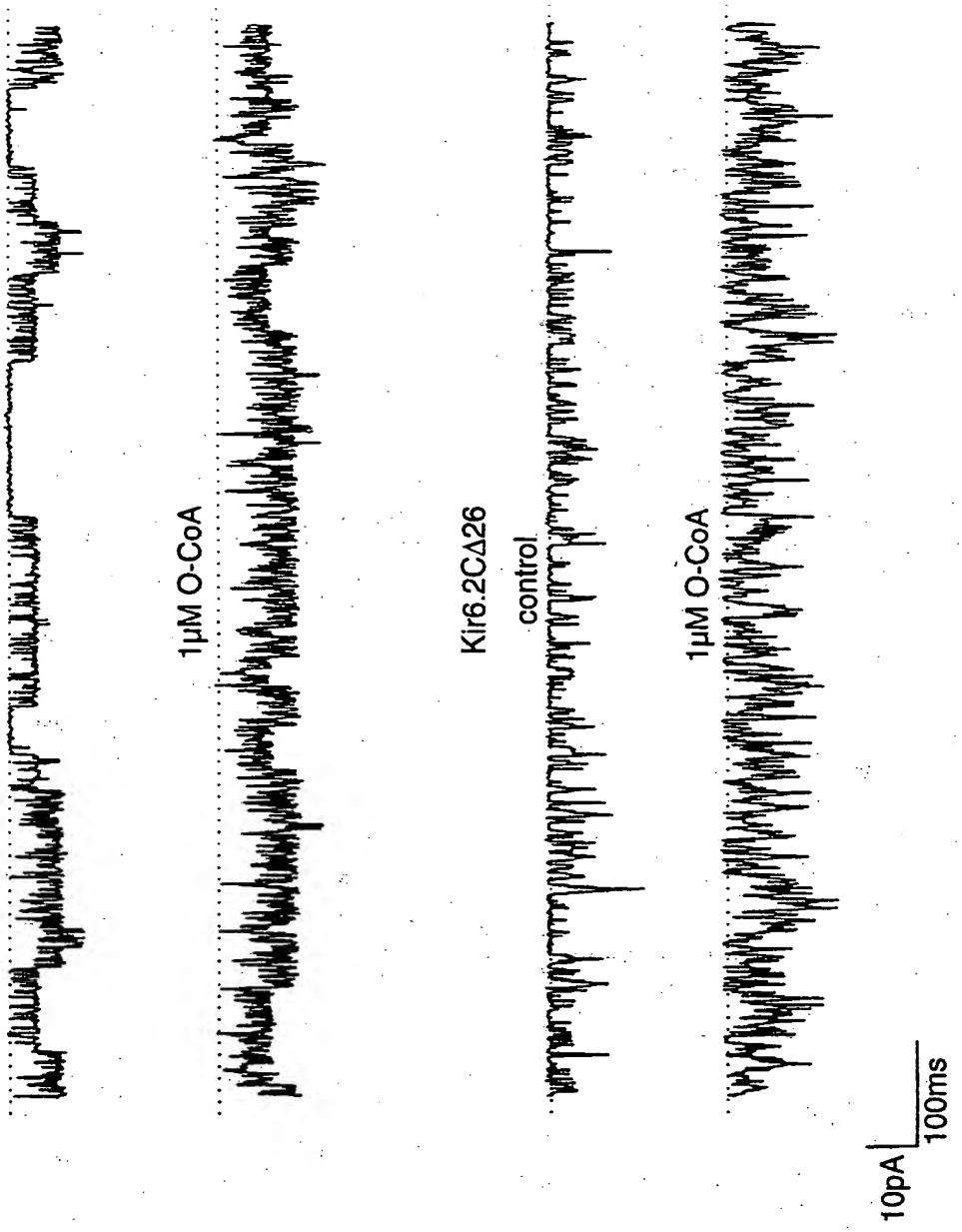
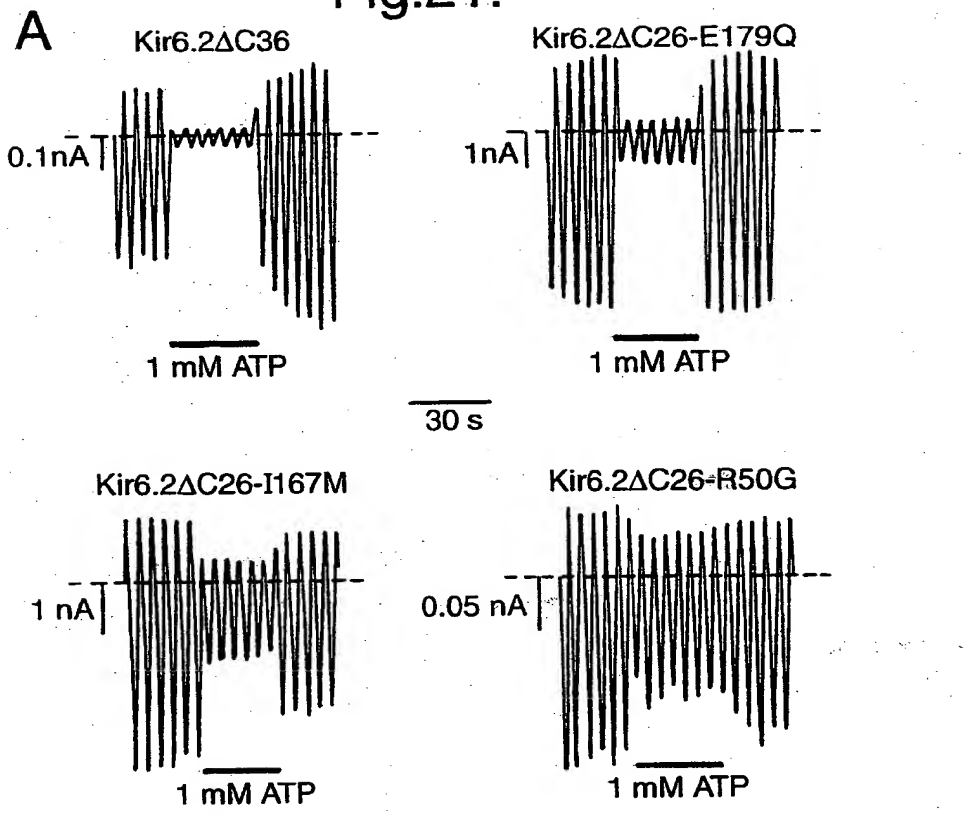

Kir6.2+SUR1
control
1μM O-CoA
Kir6.2CΔ26
control
1μM O-CoA
10pA
100ms

Fig.21.


A
Kir6.2ΔC36
0.1nA
1 mM ATP
Kir6.2ΔC26-E179Q
1nA
1 mM ATP
30 s
Kir6.2ΔC26-I167M
1 nA
1 mM ATP
Kir6.2ΔC26-R50G
0.05 nA
1 mM ATP

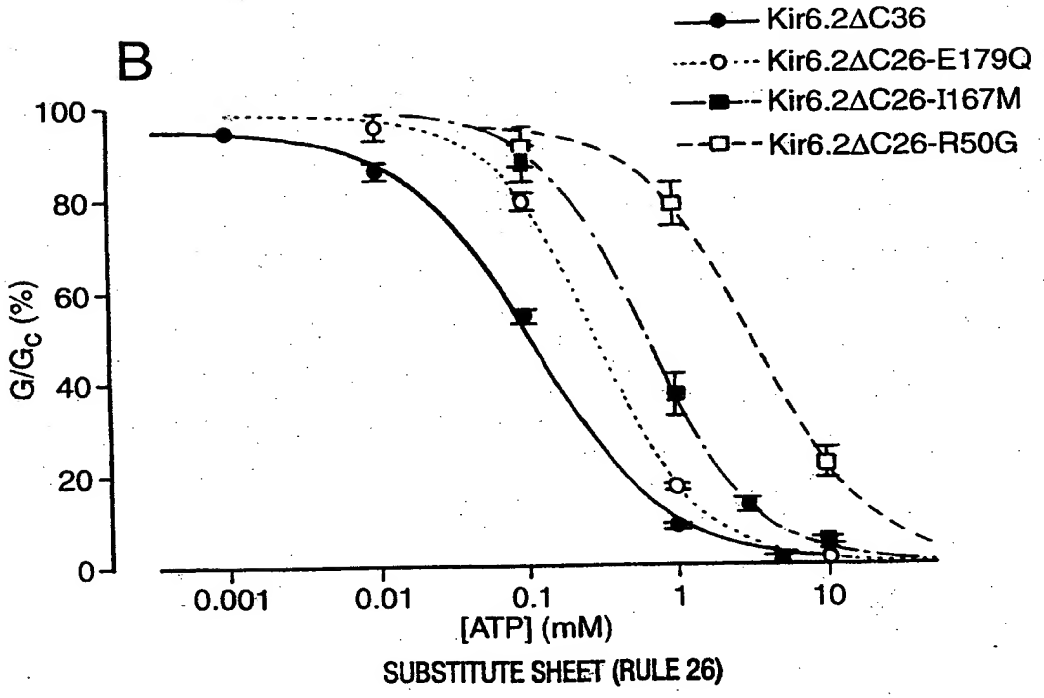
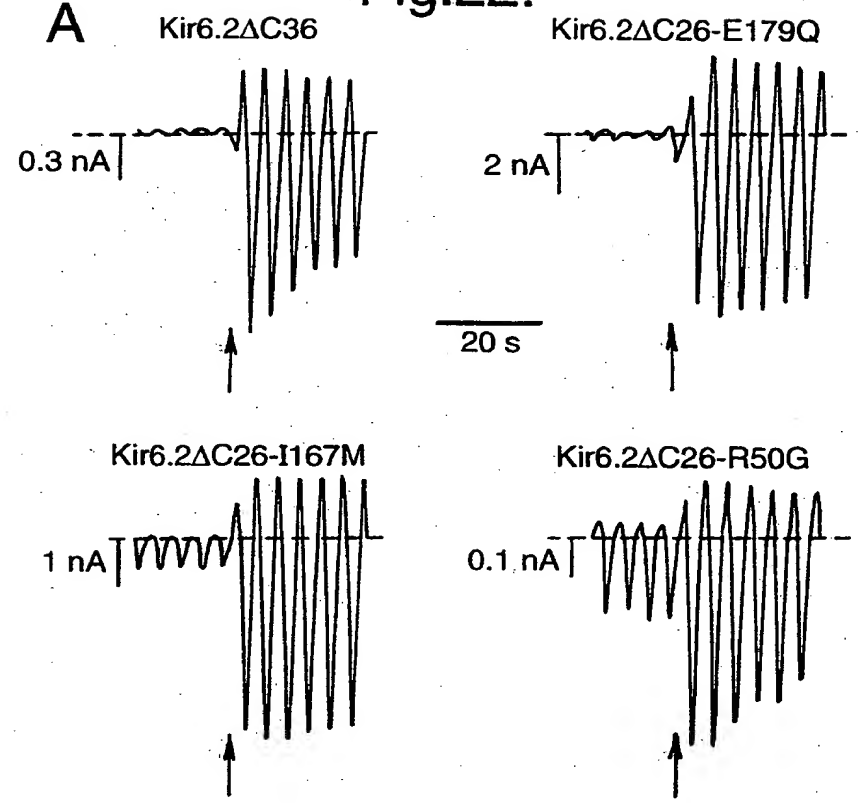

B
Kir6.2ΔC36
Kir6.2ΔC26-E179Q
Kir6.2ΔC26-I167M
Kir6.2ΔC26-R50G
100
80
60
40
20
0
G/Gc (%)
0.001
0.01
0.1
1
10
[ATP] (mM)

Fig.22.


A
Kir6.2ΔC36
Kir6.2ΔC26-E179Q
0.3 nA
2 nA
20 s
Kir6.2ΔC26-I167M
Kir6.2ΔC26-R50G
1 nA
0.1 nA

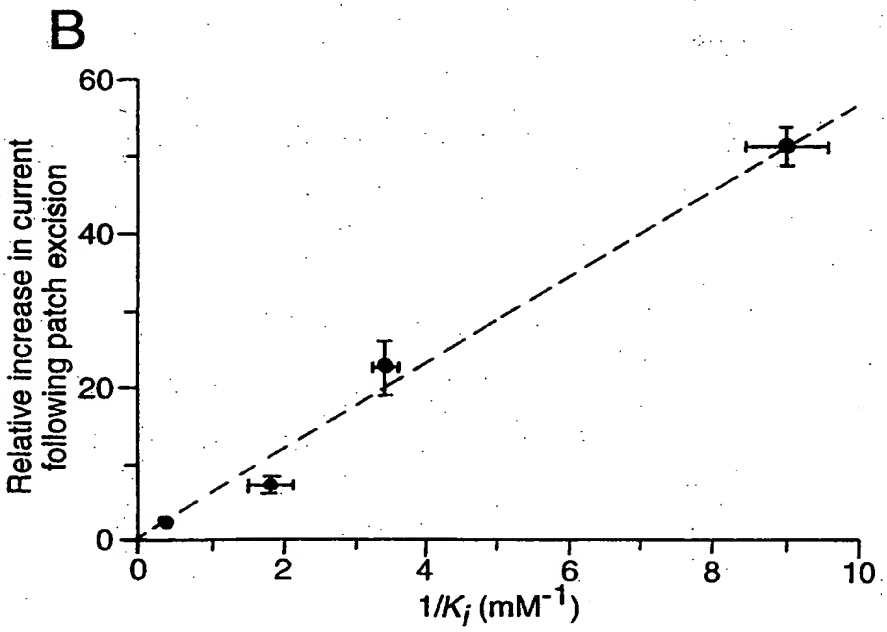
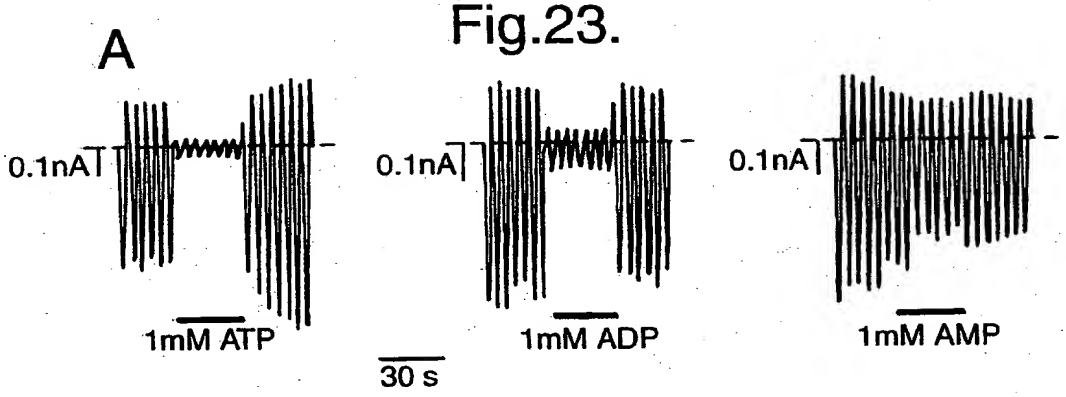
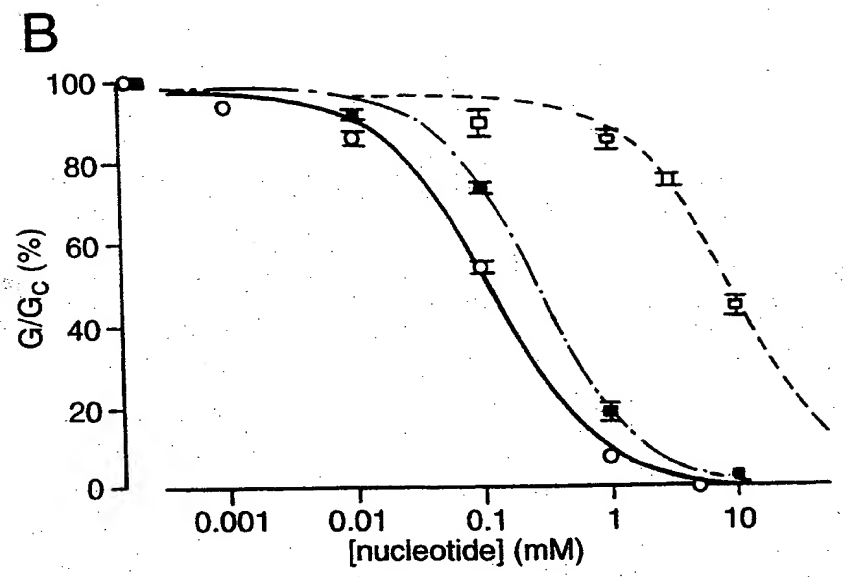

B
Relative increase in current following patch excision
60
40
20
0
0
2
4
6
8
10
1/Ki (mM-1)

Fig.23.


A
0.1nA
1mM ATP
0.1nA
1mM ADP
0.1nA
1mM AMP
30 s


B
100
80
60
40
20
0
G/Gc (%)
0.001
0.01
0.1
1
10
[nucleotide] (mM)

INTERNATIONAL SEARCH REPORT

Interna' d Application No
PCT/GB 98/00285

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/705 C12N5/10 C12Q1/68 C07K16/18
G01N33/68 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	INAGAKI N ET AL: "CLONING AND FUNCTIONAL CHARACTERIZATION OF A NOVEL ATP-SENSITIVE POTASSIUM CHANNEL UNIQUITOUSLY EXPRESSED IN RAT TISSUES, INCLUDING PANCREATIC ISLETS, PITUITARY, SKELETAL MUSCLE, AND HEART" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 11, 17 March 1995, pages 5691-5694, XP002021479 see the whole document --- -/--	1,6-9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 May 1998	16/06/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gurdjian, D

1

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INTERNATIONAL SEARCH REPORT

Internat I Application No
PCT/GB 98/00285

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	AMMALA C ET AL: "The sulphonylurea receptor confers diazoxide sensitivity on the inwardly rectifying K+ channel Kir6.1 expressed in human embryonic kidney cells." J PHYSIOL (LOND), AUG 1 1996, 494 (PT 3) P709-14, ENGLAND, XP002066564 see the whole document	1,6-9
A	INAGAKI N ET AL: "RECONSTITUTION OF /KATP: AN INWARD RECTIFIER SUBUNIT PLUS THE SULFONYLUREA RECEPTOR" SCIENCE, vol. 270, no. 5239, 17 November 1995, pages 1166-1170, XP002030881 see the whole document	1-9
P,X	WO 97 18308 A (WELLCOME TRUST LIMITED AS TRUS ;ASHCROFT FRANCES (GB); SAKURA HIRO) 22 May 1997 cited in the application see abstract; claims 1-23; figures 9,10; example 9	1-23
P,X	TUCKER SJ ET AL: "Truncation of Kir6.2 produces ATP-sensitive K+ channels in the absence of the sulphonylurea receptor." NATURE, MAY 8 1997, 387 (6629) P179-83, ENGLAND, XP002066565 see the whole document	1-8, 10-17
T	LORENZ E ET AL: "Evidence for direct physical association between a K+ channel (Kir6.2) and an ATP-binding cassette protein (SUR1) which affects cellular distribution and kinetic behavior of an ATP-sensitive K+ channel." MOL CELL BIOL, MAR 1998, 18 (3) P1652-9, UNITED STATES, XP002066566 see the whole document	1-8, 10-17

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna .l Application No
PCT/GB 98/00285

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9718308 A	22-05-1997	AU 7583296 A	05-06-1997